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DAILY AND TIDAL PATTERNS OF ACTIVITY IN INDIVIDUAL FIDDLER CRABS (GENUS UCA) FROM THE WOODS HOLE REGION[1]

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Fiddler crabs, *Uca* spp., and the shore crab, *Carcinus maenas,* possess persistent daily rhythms of color change (Abramowitz, 1937; Powell, 1962); thus, they are no exception to the proposition that circadian[2] rhythmicity is ubiquitous in organisms. Moreover, these same crabs may simultaneously exhibit rhythmic physiological adjustments to the tidal cycles of their littoral habitat. Persistent tidal rhythms have been reported in the color change of *Uca* (Brown *et al.,* 1953; Fingerman, 1960) and in the motor activity and metabolic rate of both *Uca* and *Carcinus.*

Edwards (1950) first examined the motor activity of the fiddler crabs, *U. pugilator, U. pugnax* and *U. minax.* He stated that all three species tended to be dark-active, with the exception of at least one *U. pugilator* which exhibited a bimodal daily pattern of activity occurring at sunrise and sunset. He reported a good correlation between the forms of the daily patterns of motor activity and oxygen consumption. It is worth remarking that all of his crabs were maintained in the laboratory for unspecified durations prior to recording.

Brown *et al.* (1954) recorded continuously the oxygen consumption of *U. pugnax* and *U. pugilator* in constant conditions. They reported the simultaneous presence in each species of both daily (24-hour) and semi-diurnal tidal (12.4-hour) rhythms. In discussing the metabolic rhythms in *U. pugnax,* Webb and Brown (1958) later reported that the correlation between rhythms in parallel samples of crabs tended to fall off after about seven days in the laboratory. This indicated loss of phase synchrony or rhythm among the crabs.

Bennett *et al.* (1957) demonstrated the presence of an overt, semi-diurnal

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[1] This research was aided by a contract between the Office of Naval Research and Northwestern University (1228-30) and grants from the National Institutes of Health (GM 07405) and the National Science Foundation (G-15008).

[2] In this paper circadian (etymologically, about a day) rhythms are regarded as those persistent rhythms which can be entrained by the 24-hour cycle in natural conditions. These rhythms are distinguished on an ecological basis from the semi-diurnal tidal and diurnal tidal rhythms which, although they have a period length of about a day, are entrained by a tidal cycle in natural conditions.

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tidal rhythm of spontaneous motor activity in *U. pugnax* in constant conditions. After about eight or nine days the average tidal pattern of the sample became less distinct, indicating again the loss of phase synchrony or rhythm among individual crabs. In this experiment the period length of the rhythm of activity was in some instances significantly longer than the semi-diurnal tidal period of 12.4 hours (Enright, 1965).

It was reported (Barnwell, 1963) that *U. maracoani* possessed an overt semi-diurnal tidal rhythm of motor activity which persisted for at least 11 days in constant low illumination and followed the actual changes in the periodicity of the tides at the beach of collection. *U. mordax,* maintained under the same conditions, possessed both daily and semi-diurnal tidal rhythms in motor activity.

In *Carcinus maenas* semi-diurnal tidal rhythms were found to persist for five or six days in spontaneous motor activity (Naylor, 1958) and oxygen consumption (Arudpragasam and Naylor, 1964). Both processes exhibited diurnal inequalities in the heights of the tidal peaks. For motor activity the greatest activity occurred during the nocturnal tidal peak, and for oxygen consumption, during the daytime tidal peak.

Blume *et al.* (1962) confirmed the findings of Naylor on the simultaneous occurrence of two periodic components with tidal and daily frequencies in the motor activity of *C. maenas.* In their studies the recording period was six or seven days.

In the foregoing summary, the short-term character of the experiments has been noted. Generally the curtailment of recording was due to the apparent warping or diminution of the tidal component after about a week in laboratory conditions. A second characteristic of these studies, perhaps related to the first, is that practically all of the published figures of tidal rhythms in these crabs have been *averages* of a few to many individual recordings. As the authors of these figures have usually remarked, the apparent breakdown of the average tidal pattern may be due in at least some cases to the loss of phase synchrony or divergence of pattern among individuals within the sample.

The purpose of the present study was to obtain information on the rhythms of individual crabs recorded continuously over longer intervals than in the preceding reports. Single specimens of *U. minax, U. pugilator* and *U. pugnax* were maintained in actographs for periods ranging up to 47 days. Recordings were made both in constant illumination and in natural cycles of daylight and darkness.

MATERIALS AND METHODS

The specimens of *U. pugnax* and *U. pugilator* were collected on July 10 and 11, 1963, at the Chapoquoit marsh on the southwestern perimeter of West Falmouth Harbor. West Falmouth Harbor is located on Buzzards Bay about eight kilometers north of Woods Hole, Massachusetts. The *U. minax* were collected at Wild Harbor, which is about four kilometers north of West Falmouth Harbor. Collections were made on July 3, 5 and 11 and August 9, 1963. The crabs were returned to the laboratory and placed in actographs within a day of collection.

The actographs were similar to those used in earlier studies on *Uca* activity (Bennett *et al.,* 1957; Barnwell, 1963). Crabs were placed individually with about 10 mm. of sea water in aluminum tipping pans with transparent plastic covers. One end of each pan rested on a knife edge and the other end was suspended by a thread running to a spring scale recording system. Tipping of the pan caused by movements of the crab was translated through the spring scale into horizontal excursions of a pen on a moving strip of paper. By using a series of spring scales and pens the activity of as many as seven crabs could be recorded at once on the same strip of paper.

The actograph tracings were analyzed by measuring the duration of activity in each hour and expressing it as most nearly occupying either 0, 25, 50, 75, or 100% of the hour. All activity was recorded in terms of Eastern Daylight Time, EDT.

Six actograph assemblies, recording from four to seven crabs apiece, were in simultaneous operation. Three of the assemblies, each for a different species, were maintained under constant illumination with an intensity of between three and four foot-candles at the level of the actograph pans. During the study room temperature ranged from 23.0° to 26.5° C. without obvious daily variation. Additional recordings were made in an adjacent darkroom where illumination was constant at an intensity well below one foot-candle. Crabs maintained under both light intensities will be referred to as being in constant illumination, symbolized by LL.

In the basement of a nearby building the three remaining actograph assemblies, each with a different species, were placed in front of a northward-facing window. The lower half of the window was covered with white paper so as to shield the crabs from outside movements. On any day the maximum light intensity to which the crabs were exposed ranged from about 45 foot-candles to five foot-candles for the most shaded pans. Room temperature between July 21 and Aug. 26 exhibited a slight daily variation with an average low of 22.6° C. and an average high of 23.6° C. The extreme temperatures during this period were 21° and 24° C. These crabs will be referred to as being in natural illumination, symbolized by LD.

In order to keep the crabs for extended periods it was necessary to change the water in the tipping pans at intervals of two or three weeks. This process required only a minute or so for each crab. Changing of the water was sometimes followed by a temporary increase in the level of activity, but the timing of the pattern did not appear to be altered. The crabs were not fed during the experiments.

The times of tide used for both collecting sites are the predicted values published by the U. S. Coast and Geodetic Survey for West Falmouth Harbor. The times are obtained by applying correction factors to the tidal schedule supplied for Newport, R. I. In the absence of a correction factor for Wild Harbor, the use of the West Falmouth Harbor corrections appeared to be justified, since these corrections were essentially identical with those for two locations on the shore of Buzzards Bay just to the north of Wild Harbor.

Some small but statistically significant mean differences were found when the predicted times of high and low water at Newport were compared with the actual recorded times, which were later obtained from the USCGS. During the period of the experiments, July and August, 1963, the observed times of high water occurred at 8.7 ± 11.4 minutes (mean and standard deviation) later than the predicted times. For low water during the same period the observed times were 7.5 ± 22.2 minutes earlier than the predicted times.

RESULTS

Individual recordings have been selected to illustrate the general types of patterns obtained in each species in LD and LL. For each group is listed the total number of crabs which were studied.

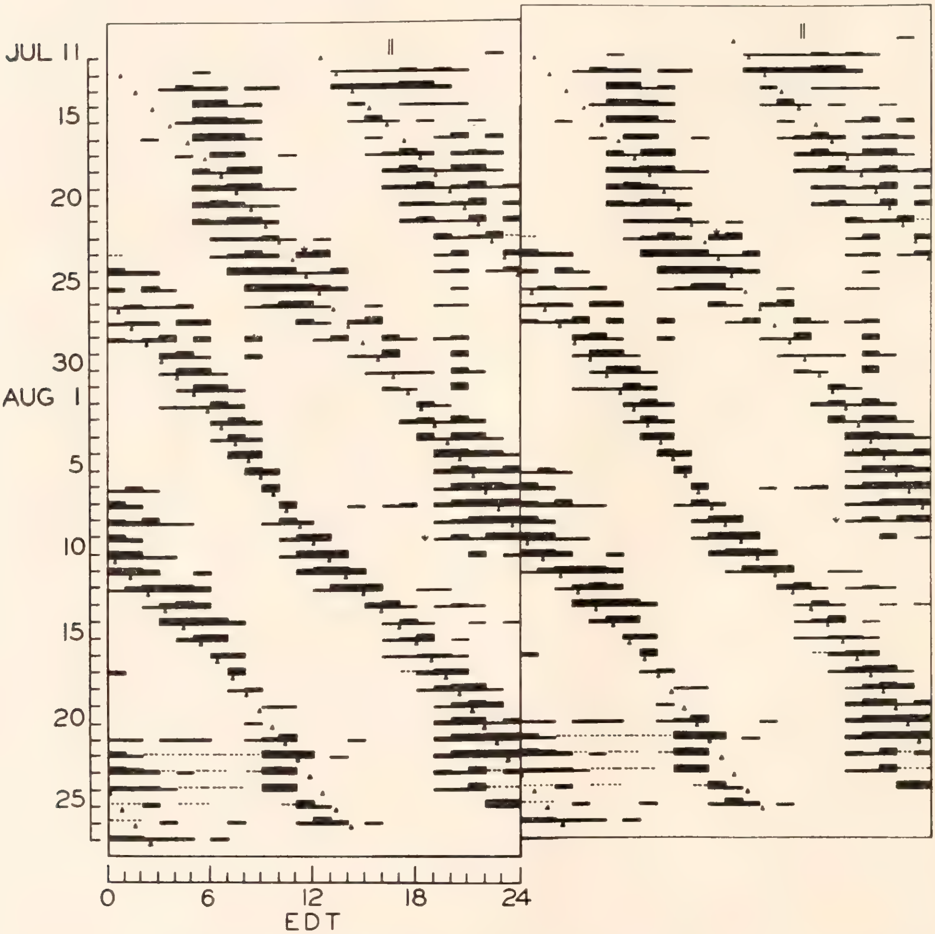

FIGURE 1. Activity pattern of a male *U. minax* in LD. The graph has been reproduced twice, with the right-hand graph displaced upward by one day, in order to facilitate the visualization of the drift of activity peaks across the solar day. The height of the blocks indicates the per cent of the hour during which activity was recorded, either 25, 50, 75, or 100 per cent. Parallel vertical lines at the beginning of the record mark the time of collection. Small triangles indicate the predicted times of high tide at the beach of collection. Small arrows point to times when water in the tipping pans was changed. Broken lines indicate failure of the recording system. These conventions are used for all figures.

U. minax—LD (7 crabs)

Crab 1 (Fig. 1). This male crab exhibited an overt semi-diurnal tidal rhythm which persisted through 46 complete days of recording. The frequency of the rhythm was very close to tidal frequency. A 24-hour component was expressed both as an initial suppression of tide-related activity between 24:00 and 05:00 (July 12 to about July 21) and as an enhancement of activity when the tidal peak occurred between 19:00 and 21:00 (July 17 to 22, Aug. 2 to 9, 17 to 24); at both times of day the initiation of activity tended to conform for several days to the time of sunrise or sunset. The tendency for the tidal peak to be suppressed during the morning hours disappeared with passing time, as subsequent tidal peaks were obvious between 24:00 and 05:00 (from July 24 to 30, Aug. 6 to 15, and Aug. 21 to 27). Daily activity related to sunset depended primarily on the presence of the tidal peak for its expression. Occasionally, a tendency for activity at sunset was expressed by isolated bursts of activity not associated with tidal peaks. However, the periods of greatest activity took place at semi-monthly (15-day) intervals when tidal activity coincided with sunset.

Crab 2 (Fig. 2). This is the first portion of the record of a female crab in which the tidal rhythm was evident for 40 days of recording. The record demonstrates that suppression of the tidal peak between 24:00 and 05:00 need not always occur when the crab is first removed from the beach to the experimental condition of LD.

Crab 3 (Fig. 3). This male attempted to molt between 09:00 and 10:00 on Aug. 7. The semi-diurnal pattern persisted with a high level of activity up to the time of molt. During the initial 11 days of recording, the activity peak in the first half of the day appeared to conform to a 24-hour schedule and effectively masked any tide-related activity.

U. minax—LL (14 crabs)

Crab 4 (Fig. 4). This male crab was collected on July 5 for a preliminary experiment. Its activity was recorded in LL at three to four foot-candles until 22:00 on July 10 when it was transferred to the lower light intensity of the

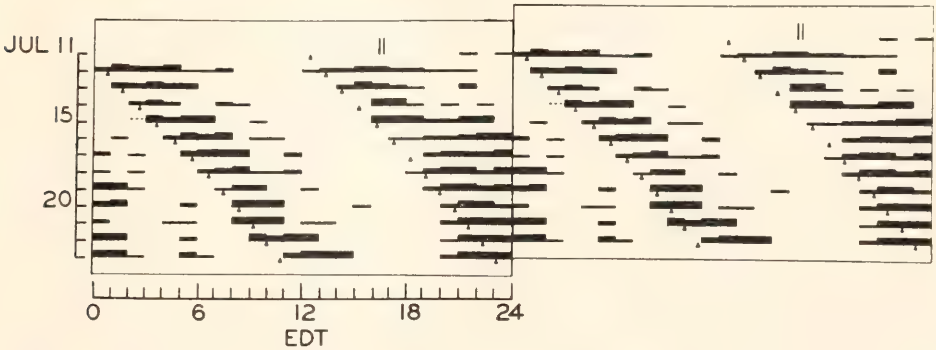

FIGURE 2. Activity pattern of a female *U. minax* in LD.

adjacent darkroom. Initially the semi-diurnal tidal rhythm bore the same relationship to the time of tide as that already observed for the crabs in LD, that is, initiation of activity within an hour or two of the time of high tide. However, the period of the rhythm in LL was clearly longer than 12.4 hours. Furthermore, there was not the pronounced suppression or enhancement of activity related to the time of sunrise or sunset that was evident in the crabs in LD. The tidal pattern persisted for at least five weeks. During the last week of recording it appeared to undergo a transition from a bimodal to a predominantly unimodal daily pattern, in which activity occurred mainly in the latter half of the day.

Of the 14 *U. minax* kept in LL, ten possessed a distinct semi-diurnal tidal rhythm, the period of which exceeded the natural mean tidal period of 12.4 hours. There were individual differences in the period length of the rhythm, and in some cases the period was not constant but increased gradually. Three of the remaining four crabs possessed tidal rhythms, but the activity became so diffuse that it was not possible to determine from inspection if the period length deviated from the natural tidal period. The pattern of the fourth crab is described next.

Crab 5 (Fig. 5). This crab was an ovigerous female which was collected on July 3 for a preliminary experiment. Its activity was recorded in LL at three to four foot-candles until 10:45 on July 8 when it was transferred to the lower light intensity of the photographic darkroom. By July 12, when water was replaced in the tipping pan, the larvae had emerged. Most striking in this case is the gradual disappearance of the tidal component and the eventual domination of the daily pattern by a major peak. Initially, activity was tide-related, with

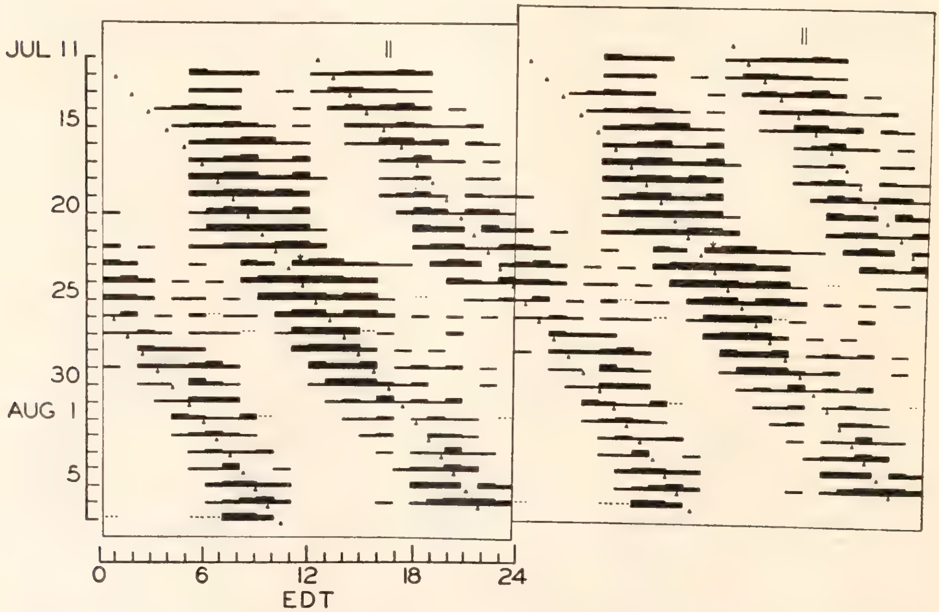

FIGURE 3. Activity pattern of a premolt male *U. minax* in LD. The crab was found attempting to molt at 10:00 on Aug. 7.

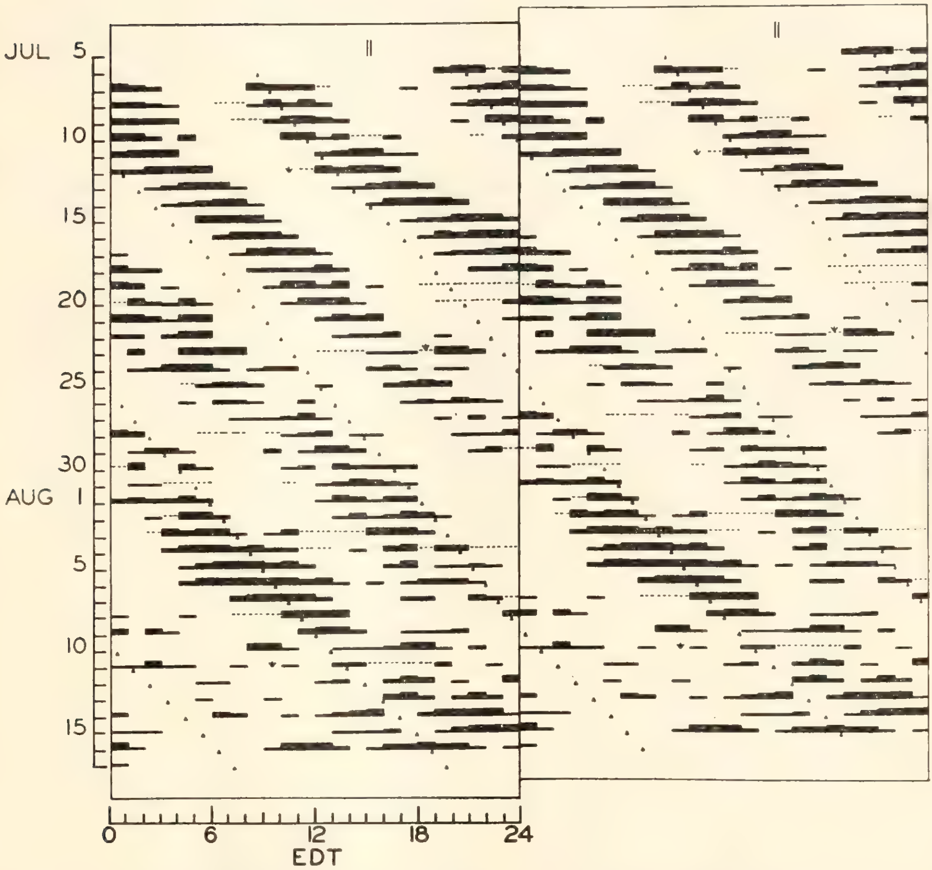

FIGURE 4. Activity pattern of a male *U. minax* in LL. At 22:00 on July 10 this crab was transferred to the lower light intensity of the photographic darkroom.

the nocturnal tidal peak being the more conspicuous of the two peaks. The nocturnal tidal peak appeared to drift into the daylight hours but then disappeared after a final peak at 15:00 on July 26. The original daytime tidal peak (07:00 to 10:00 on July 6) appeared to drift across the day and merge with a diffuse band of nocturnal activity. In addition to the two tidal peaks and the broad band of daily activity which eventually emerged, there seemed to be other component bands (*e.g.,* activity centered on 09:00 to 10:00 from July 26 to July 31, then on 11:00 to 12:00 from Aug. 1 to Aug. 3) which contributed to a generally complex pattern.

U. pugilator—LD (6 crabs)

Crab 6 (Fig. 6). Both an accurate semi-diurnal tidal rhythm and a daily rhythm, consisting of pronounced activity after sunset, were present for the 29 days of recording. For this species a 24-hour component of activity at sunset was evinced as a daily recurrence and was not as dependent upon the phase of tidal activity for its expression as in *U. minax*. In the records of three of the five other *U. pugilator* in LD the nocturnal activity was equally conspicuous.

U. pugilator—LL (6 crabs)

Crab 7 (Fig. 7). The semi-diurnal pattern of this crab could be traced for about two weeks. Thereafter, one tidal peak was obscured as it moved into a band of activity centered at 02:00 from July 21 to 28. The second tidal peak scanned across the daytime hours. It could perhaps be traced for somewhat longer than the other peak, but by the late afternoon of July 30 to 31 it had faded.

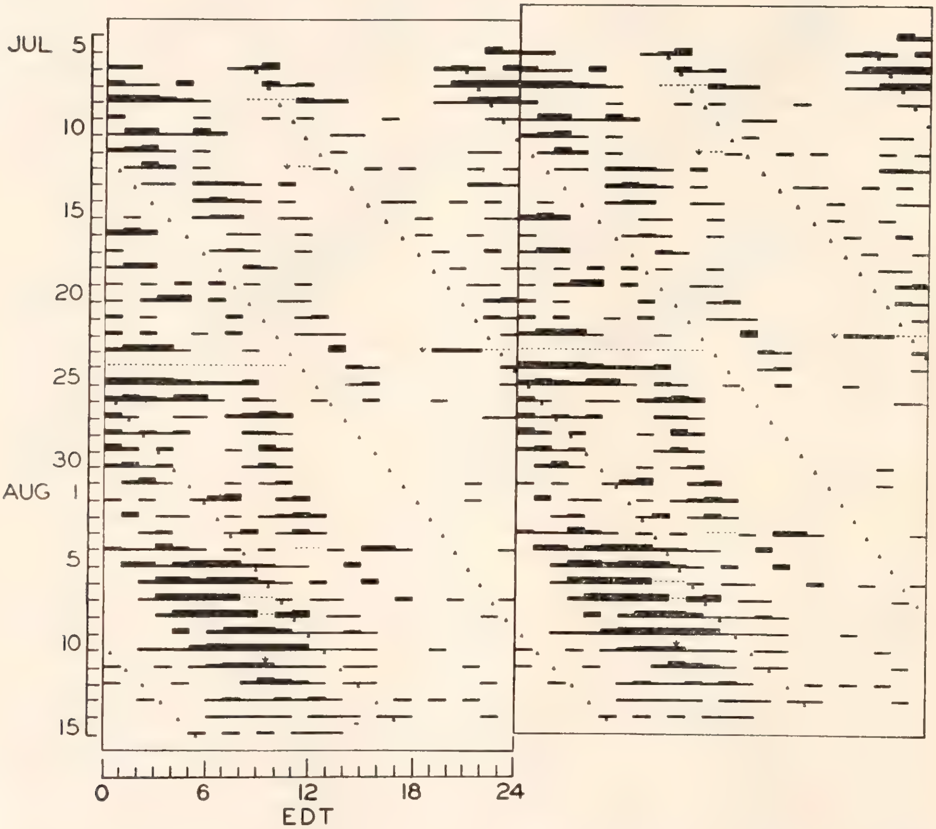

FIGURE 5. Activity pattern of a female *U. minax* in LL. At the time of collection, on July 3, this crab was ovigerous. At 10:45 on July 8 it was transferred to the lower light intensity of the photographic darkroom.

Crab 8 (Fig. 8). Conspicuous in this crab is the augmentation, early in the recording period, of a single major daily peak of activity. This major peak was initially a nocturnal one, and its period length appeared from inspection to be less

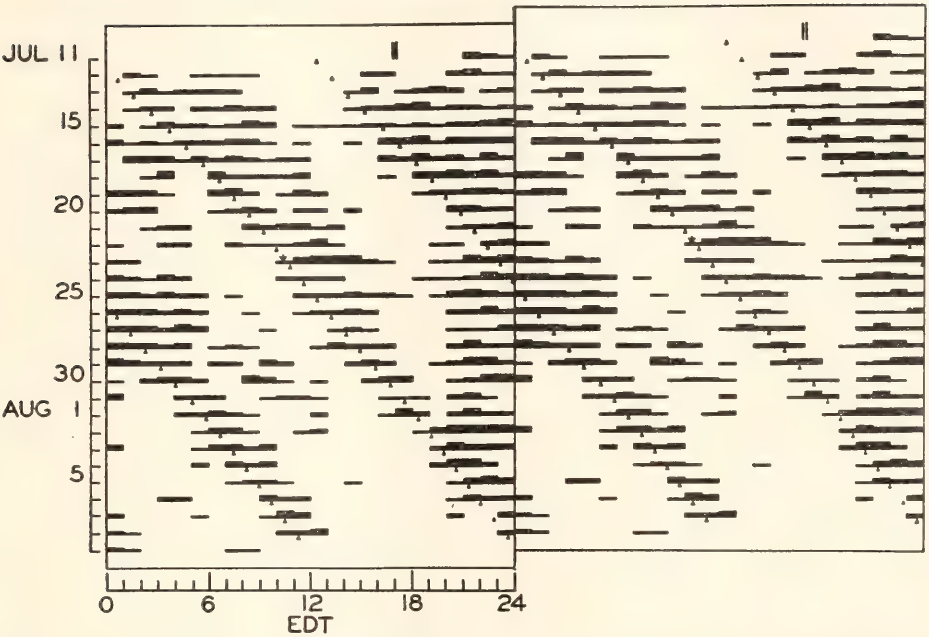

Figure 6. Activity pattern of a male *U. pugilator* in LD.

than the diurnal-tidal (24.8-hour) period. There is some suggestion that one tidal peak persisted through the daytime hours with close to tidal frequency. A transition from a bimodal to an essentially unimodal daily pattern of the same

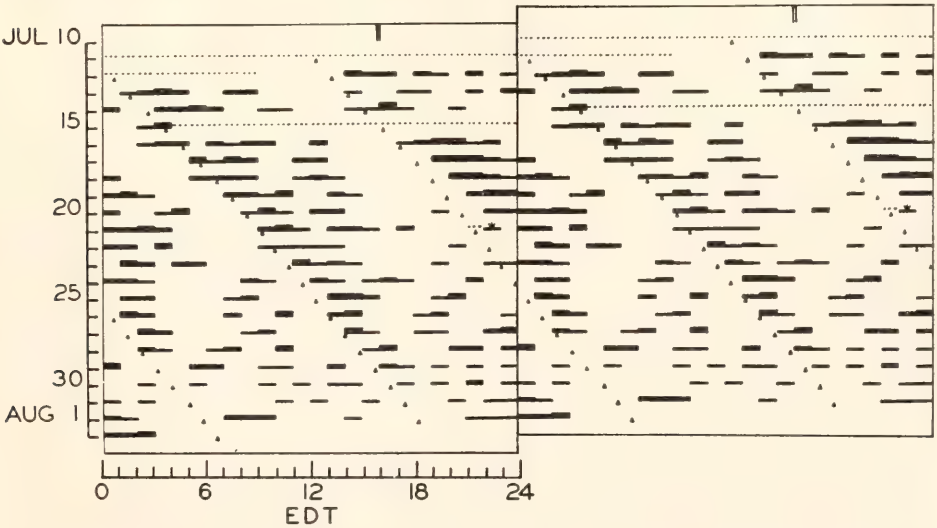

Figure 7. Activity pattern of a male *U. pugilator* in LL.

character as the one described in this case was observed in another crab of the same group.

U. pugnax—LD (7 crabs)

Crab 9 (Fig. 9). This crab exhibited a conspicuous semi-diurnal tidal rhythm which persisted with good accuracy for the 30 days of recording. In this case the expression of a 24-hour component of activity depended mainly on the presence of the tidal peak. This pattern is representative of five others which were obtained from *U. pugnax* in LD, although there were individual differences in the strength of expression of the daily component.

Crab 10 (Fig. 10). This pattern is described because of its variance from the quite consistent patterns recorded for the six other *U. pugnax* in LD. On July 16 the evening tidal peak suddenly increased its period and scanned across the day until it reached the time when the second tidal peak would be occurring. At this point (about 11:00 on July 23 to 24) it appeared to resynchronize to a tidal schedule. At the same time that the deviant component began to merge and resynchronize, a band of activity reappeared (02:00 on July 23) on the tide which it had originally followed. The pattern suggests that this crab, like the six other *U. pugnax*, maintained through the recording period a basic tidal frequency, but in this case the tidal rhythm was sometimes unexpressed and upon it was superimposed a component of a different frequency.

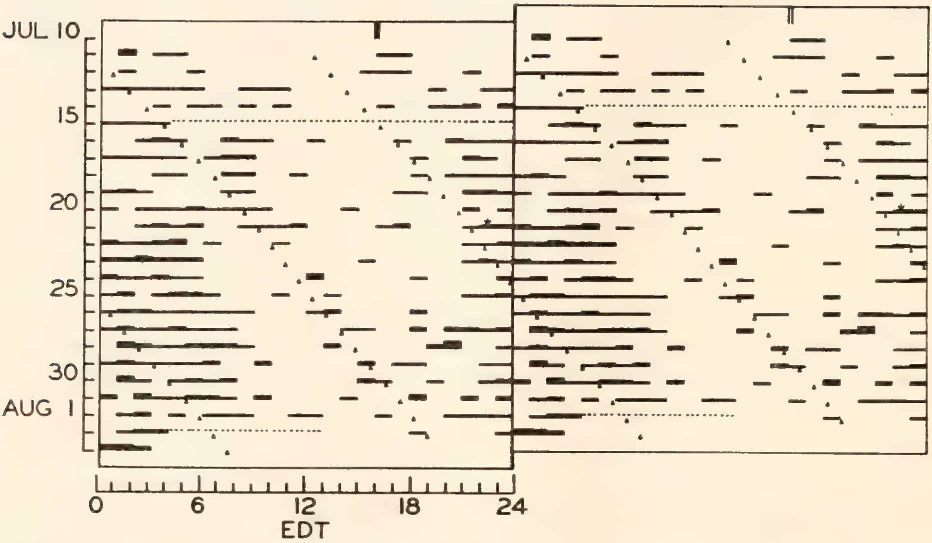

FIGURE 8. Activity pattern of a male *U. pugilator* in LL.

Crabs 11 and 12 (Fig. 11). Portions of the records of these two *U. pugnax* were selected to indicate phase differences within the sample. Crab 11 (Fig. 11 A) tended to become active about an hour before the time of high tide, while the major period of activity for crab 12 (Fig. 11 B) was initiated about four to five hours after the time of high tide. In both patterns a 24-hour rhythm was

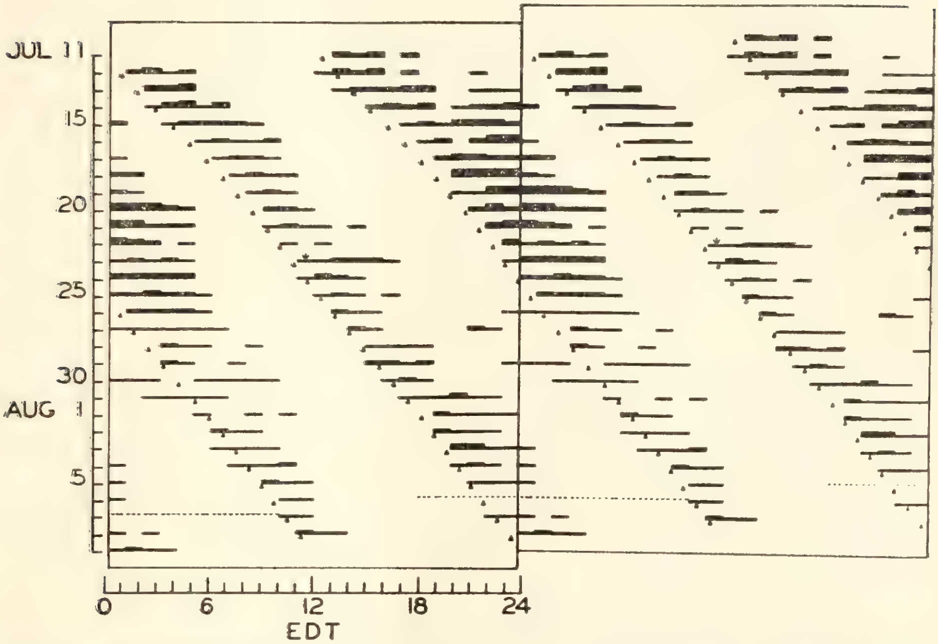

FIGURE 9. Activity pattern of a male *U. pugnax* in LD. The crabs of this group were collected on July 10.

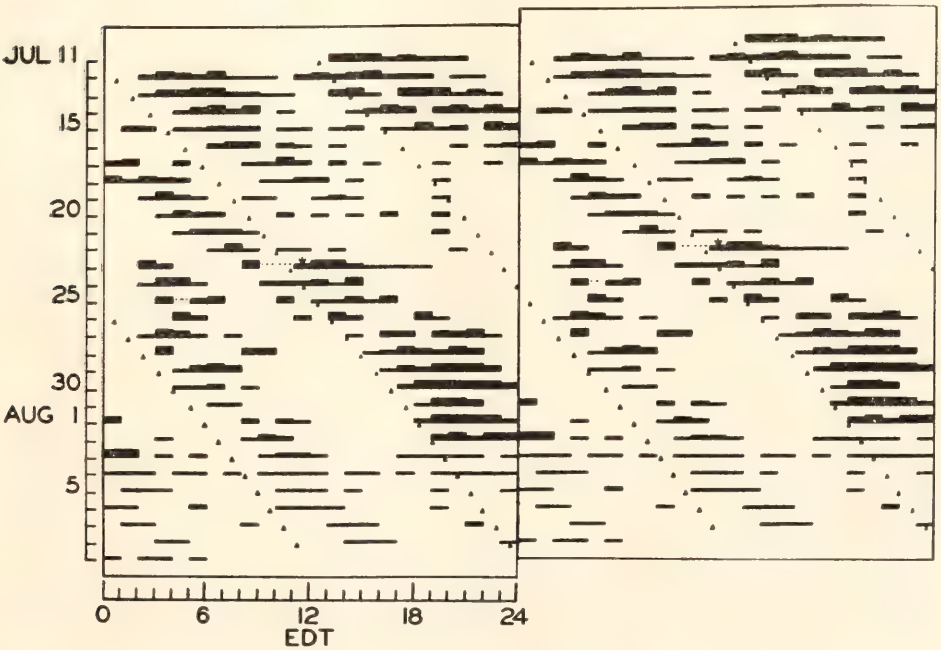

FIGURE 10. Activity pattern of a male *U. pugnax* in LD.

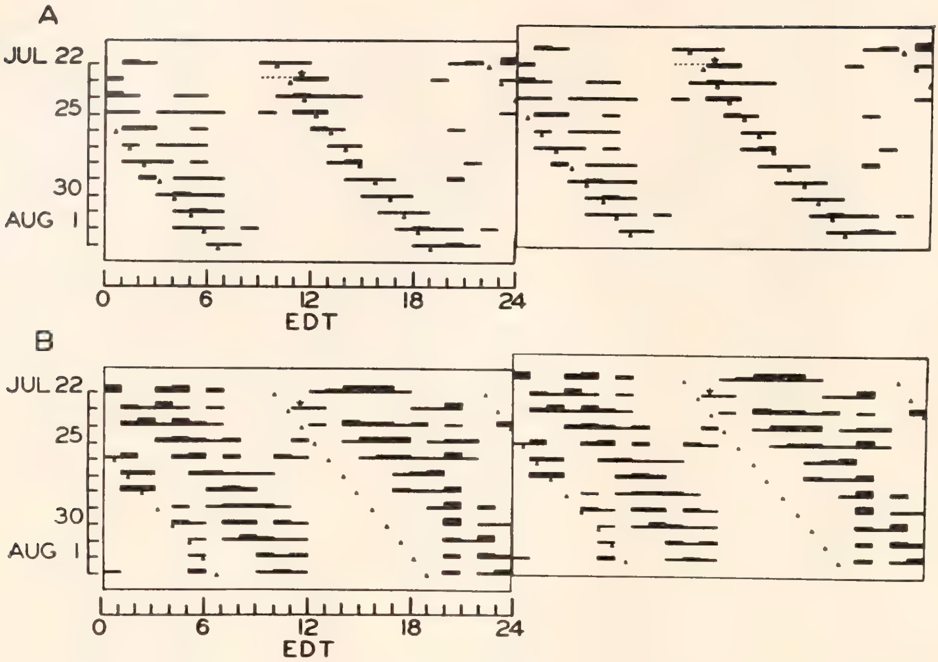

Figure 11 A and B. Activity patterns of two male *U. pugnax* in LD.

superimposed on the tidal pattern as bursts of activity between 04:00 and 06:00 and between 20:00 and 21:00. In crab 9 (Fig. 9) the initiation of activity relative to the time of tide is intermediate between those of the two crabs in Figure 11. Thus, it is evident that in the same experimental conditions crabs of the same sample may exhibit persistent phase differences among themselves.

U. pugnax—LL (4 crabs)

Crab 13 (Fig. 12). The semi-diurnal rhythm persisted until a few days before the crab's death on Aug. 2. Because the activity is diffuse, it is difficult to determine from inspection whether or not the tidal rhythm increased in period length. Unfortunately, none of the *U. pugnax* in constant illumination survived beyond Aug. 3.

Discussion

This study demonstrates clearly that an overt tidal rhythm of motor activity may persist for as long as five weeks in constant conditions, and for even longer in natural illumination.

At the same time, it can be seen that a single daily peak may come to dominate the activity pattern, either early (crabs 5 and 8) or later (crab 4) during the exposure to LL. Recordings in LD indicate that in their day-related—as distinct from tide-related—activity *U. pugilator* and *U. minax* are primarily dark-active.

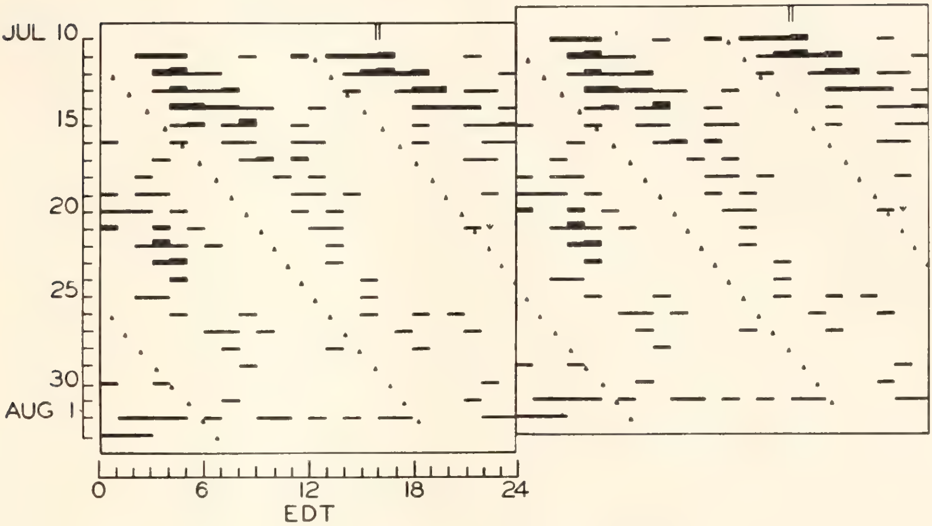

FIGURE 12. Activity pattern of a male *U. pugnax* in LL.

Since the major peak observed in LL was of nocturnal origin in two of the cases, and since its period tended to be less than diurnal tidal (24.8-hours), it seems likely that it represents a circadian component. Several years ago Prof. Hermann Schöne (personal communication) observed a circadian rhythm in the motor activity of *U. pugilator.* The rhythm persisted for many months in constant illumination with a period which was a function of light intensity. The period length increased as the logarithm of light intensity increased, a relationship which has been observed frequently in dark-active animals (Hoffmann, 1965).

For all three species a daily component was more pronounced in natural illumination. For *U. pugnax* it consisted of increased activity between sunset and sunrise (20:00 to 05:00). This rhythm has its counterpart in the persistent daily rhythm of metabolic rate of *U. pugnax* in constant conditions (Brown *et al.,* 1954; Webb and Brown, 1958). The average daily metabolic variation, obtained from five summers of recording, was characterized by a rapid increase in rate at about 20:00, a major maximum at 05:00, and a gradual decline to a minimum rate at 17:00 (Brown, 1960, Fig. 6 C, with times converted from EST to EDT). The ecological significance of the form of the persistent metabolic rhythm is also indicated by Crane's (1958) observation that for most fiddler crabs in the field the tides occurring between 12:00 and 17:00 appear to be disadvantageous.

The persistence of the tidal rhythm is of interest with regard to the timing of semi-monthly (15-day) cycles. It has been pointed out (Brown *et al.,* 1953) that a semi-monthly cycle could be timed by the periodic interference of simultaneously occurring semi-diurnal tidal and daily cycles. The present study provides a graphic demonstration of just such an accurate and persistent tidal rhythm as would be required for the mechanism of periodic interference. In the case of *U. minax* (Fig. 1) three accurate semi-monthly periods were measured off in non-tidal conditions.

With respect to the mean tidal period of 12.4 hours, the accuracy of the tidal rhythm in natural illumination is quite remarkable; it is greater than the accuracy which is usually attributed to the proposed models of strictly endogenous oscillatory systems. It is unlikely that there occurred in the experimental setting any sort of human activity or other obvious disturbance which could have provided the crabs with time cues of a semi-diurnal tidal nature. Furthermore, the large individual differences in phase (crabs 11 and 12) and pattern (crabs 9 and 10) in crabs located side by side in the same actograph appear to rule out the possibility that some uncontrolled tidal phase synchronizer, or *Zeitgeber,* was influencing the crabs, or that the crabs were somehow influencing one another, as through vibrations.

The observation that the period length of the tidal rhythm may depart widely from 12.4 hours in LL but not in LD suggests a relationship between the accuracy of the tidal rhythm and the presence of a 24-hour light cycle. A possible explanation of this relationship is provided by two earlier findings in *Uca.* First, it has been shown that when the daily rhythm of color change is rephased by artificial light cycles, the tidal rhythms of color change (Brown *et al.,* 1953) and motor activity (Bennett and Brown, 1959) are both reset by a corresponding amount of time. Second, in constant illumination the daily rhythm of color change in several species of *Uca* (Webb *et al.,* 1954; Sandeen and Wheatley, 1962) may exceed 24 hours in period length. Given (a) some sort of coupling between daily and tidal rhythms during phase shifting and (b) a lengthening of period, or phase drift, of the daily rhythm, then a lengthening of period of the tidal rhythm is not an unexpected consequence.[3] Thus, inaccuracy of the tidal rhythm could be explained simply as a result of inaccuracy of the circadian rhythm. When phase and period of the circadian rhythm are synchronized with a natural light cycle, an accurate tidal rhythm would be permitted to express itself.

It is also possible that the tidal rhythm, of itself and apart from the circadian component, was exhibiting deviations in period length which were analogous to those of circadian rhythms. Why this should have occurred in LL but not in LD is not clear. Furthermore, the interpretation of the circadian period is itself an unresolved question. Either the circadian period in the steady-state is the same as the period of its endogenous, driving oscillator (Pittendrigh, 1960; Aschoff, 1960), or it arises through intra-organismic frequency transformation of basic clock periods, proposed by Brown to be geophysically derived, accurate solar- and lunar-day ones. The theoretical possibility of frequency transformation has been acknowledged (Klotter, 1965), and its biological significance has been discussed (Brown, 1962, 1965). At present neither of the two alternative theories has been disproved, and both account for the properties of circadian rhythms. However, the theory of external timing accounts not only for circadian rhythms but for persistent rhythms of accurate daily and lunar (tidal) frequency.

The activity pattern recorded for crab 10 (Fig. 10) appears to be a case of dissociation of components of a tidal clock system. The record was obtained in LD, assuring, presumably, that the circadian component was phase-locked and could not contribute to the dissociation. Examples have been published of a similar phenomenon in the circadian rhythms of certain arctic mammals (Pittendrigh, 1960, Figs. 10 and 11).

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[3] Experimental evidence on this point has recently been presented by Webb and Brown (1965).

There was considerable variation among the activity patterns of individual crabs. At the species level several generalizations could be made. In *U. minax* the tidal rhythm was most distinct and persistent under both conditions of illumination, and under constant illumination exhibited the greatest tendency to deviate from the tidal period. *U. pugilator* exhibited the most conspicuous component of nocturnal activity and the greatest tendency to undergo transitions from a tidal to circadian pattern in constant illumination.

Among crabs of the same species several factors have been reported to affect the expression of daily and tidal components. One of these is the exposure to tidal influence. Altevogt (1959) reported that in the field the activity of *U. tangeri* in a non-tidal pool conformed to a daily schedule, while crabs in the near-by intertidal zone carried on the same activities on a tidal schedule. Fingerman *et al.* (1958) found that the persistent melanophore rhythm of *U. pugilator* from above the high water line was predominantly a daily rhythm, while the rhythm was conspicuously tidal in crabs from the intertidal zone. Naylor (1960) reported that *C. maenas* from a non-tidal dock exhibited principally nocturnal activity in LD rather than tidal activity. Blume *et al.* (1962) also reported that in *C. maenas* either a semi-diurnal tidal cycle or a daily cycle may dominate the activity pattern.

In *C. maenas,* Naylor (1960) showed that the tidal rhythm of crabs from a tidal habitat could be suppressed by maintaining the crabs in a non-tidal aquarium for four weeks. Their activity came to resemble that of crabs from the non-tidal dock. By chilling, a tidal rhythm could be induced in these non-tidal crabs (Naylor, 1963). The experimentally demonstrated lability of the tidal rhythm in *C. maenas* suggests that under natural conditions the rhythm can be switched off and on in accordance with changes in environmental circumstances.

A second factor influencing the pattern of activity is reproductive state. Hyman (1920) reported that ovigerous females of *U. pugnax, U. pugilator,* and *U. minax* emerged from their burrows during low tides at sunset, but were not to be seen running on the beach with the males during the low tides in the daytime. In this regard it is of interest that nocturnal activity dominated the activity pattern of the ovigerous *U. minax* (Fig. 5).

From extensive observations on individual fiddler crabs of several species, Crane (1958) was able to classify their social activity into six phases. Among these phases the amount of motor activity varied widely. In the "underground" phase the crab did not emerge at all from its burrow during low tide, while in the "non-aggressive wandering" and "aggressive wandering" phases the crab left its burrow and walked almost incessantly during the period of low tide. Populations of crabs tended to pass through the sequences of phases from least to most social in semi-monthly cycles, but large individual differences were evident. Thus, a third factor contributing to individual differences in activity could be internally controlled level of social behavior.

Bliss (1960) has reported that in constant darkness the circadian rhythm of the land crab, *Gecarcinus lateralis,* had significantly greater variability in eyestalkless and intact premolt crabs than in intact intermolt crabs. In the present study only a single crab (Fig. 3) is known to have been in premolt condition. This crab exhibited in natural illumination an accurate tidal rhythm with a high level of activity up to the moment of molting. Unfortunately, there have not been more extensive reports on the effects of the premolt condition on the daily and tidal patterns of activity in fiddler crabs.

Up to the present, little has been done on the physiological control of the expression of daily and tidal components, although an experiment by Brown *et al.* (1954) is of some interest. These authors reported that eye-stalk ablation in *U. pugilator* abolished the tidal, but not the daily rhythm of metabolic rate in constant conditions. In this regard it is significant that the eyestalk is involved in the regulation of reproduction, molt, and level of motor activity, those factors which have just been discussed as contributing to differences in the expression of daily and tidal rhythms.

The author is very grateful to Prof. Frank A. Brown, Jr., for discussions of this work and for his critical reading of the manuscript.

SUMMARY

1. The motor activity of individual specimens of *U. pugnax, U. pugilator,* and *U. minax* was recorded in actographs in both constant illumination and natural cycles of daylight and darkness for periods which exceeded six weeks in some crabs.

2. In the natural light cycle crabs exhibited overt, semi-diurnal tidal rhythms of high accuracy which persisted, in the case of *U. minax,* for at least 46 days. Superimposed on the tidal rhythm was a 24-hour component which consisted generally of activity between sunset and sunrise.

3. In constant illumination the tidal rhythm in *U. minax* clearly exceeded the natural tidal period of 12.4 hours. In some crabs of this species the tidal rhythm persisted for well over a month.

4. In both *U. minax* and *U. pugilator* transitions from a tide-related pattern to an apparently circadian pattern were observed in constant illumination.

5. Evidence was presented for dissociation of components of the tidal clock system in *U. pugnax.*

6. Variations in the activity pattern occur at the species level and within species. Within species individual variation may be due to such factors as exposure to tidal influence, reproductive state, phase of social activity, and molting.

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CHEMICAL ANATOMY OF THE PERICARDIAL AND PERIVISCERAL FLUIDS OF THE STINGRAY, DASYATIS AMERICANA[1]

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The significance of the observation by Staedeler and Frerichs (1858) of "colossale Quantitaten" of urea in the blood and tissues of some skates and a shark remained obscure for four decades. In 1897 Bottazzi reported that the freezing point depressions of elasmobranch blood and of sea water were approximately equal, that is, they had approximately the same total solute particle, or osmolal, concentrations. But whereas sodium and chloride make up the bulk of solute particles in sea water, Rodier (1899) observed that urea accounted for about one-third of the solute particles of elsamobranch blood. Rodier also noted that the freezing point depression of the fish blood was generally slightly greater than that of the animal's sea-water environment, an observation which has been repeated often (Fredericq, 1904; Garrey, 1905; Bottazzi, 1906; Scott, 1913; Smith, 1936). In shallow waters, such as those generally used for collection of experimental animals, the environmental solute concentration may fluctuate depending upon (among other factors) the season, height of tide, location, depth of water, and time of day. Thus, one could expect that the freezing point depression (total osmolality) of elasmobranch blood would vary depending upon the environment (see Table I). Experiments by Scott (1913) demonstrated that the urea of the blood was not the only solute to be affected by an environmental concentration change; sharks immersed in hypo-osmotic environments lost chloride, probably *via* the gills. Smith and Smith (1931) reported that the blood of fresh-water elasmobranchs contains decidedly less urea and chloride than that of marine elasmobranchs. More recently, Hartman *et al.* (1941) found that injection of urea into the vascular system of *Raja erinacea* caused a decrease in the blood's sodium concentration. They also observed that during inanition blood urea fell and sodium became elevated. Apparently, as Smith had observed in 1936, there is a reciprocal relationship between the concentrations of salt and urea.

The first comprehensive survey of the inorganic solutes of elasmobranch blood was made by Homer Smith (1929). His data plus other data from the literature are summarized in Tables II and III. Most notable are the relatively high and somewhat variable levels of sodium and chloride in the blood. The apparently variable levels of blood calcium, magnesium and potassium may actually reflect difficulties of analysis rather than extreme variation within the group.

Many investigators have measured the blood urea nitrogen (BUN) of elasmobranchs. As one could expect from what has been said above, the values range widely: from 750 mg.% (Scott, 1913) to 1100 mg.% (Smith, 1929) in blood

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TABLE I

Published mean freezing-point values for plasma or serum samples from various elasmobranchs. Synonymies have been checked in Bigelow and Schroeder (1948, 1953). In some cases the freezing point has been calculated from data expressed as milli Osmoles/Kg.

Group	Species	Δ (° C.)*	Reference
Selachians			
	Mustelus canis	−1.79	Doolittle *et al.* (1960)
	Mustelus canis	−1.87	Scott (1913)
	Scyliorhinus caniculus	−2.06	Para (1936)
	S. stellaris	−2.3−−2.42	Bottazzi (1897)
	Scyllium sp.	−2.20	Duval (1925)
	Squalus acanthias	−1.80	Dakin (1908b)
		−1.81	Maren (1962)
		−1.83	Stone and Dewell (1962)
		−1.84	Scott (1913)
		−1.87	Rodnan *et al.* (1962)
		−1.89	Burger and Hess (1959)
		−1.90	Denis (1913)
		−2.04	Macallum (1926)
Batoids			
	Dasyatis americana	−1.64	Wynn and Bernard (1964)
	Raja sp.	−1.88	Garrey (1905)
	R. batis	−1.82	Dakin (1908a)
	R. clavata	−2.04	Para (1936)
	R. erinacea	−1.80	Chaisson and Friedman (1933–34)
	R. laevis	−1.78	Maren *et al.* (1963)
	R. ocellata	−1.73	Maren *et al.* (1963)
	R. radiata	−1.51	Dakin (1908a)
	*R. undulata***	−2.07	Para (1936)
	Trygon sp.	−2.38	Bottazzi (1897)
		−2.44	Mosso (1890)

* Data presented by Bottazzi (1897) indicate that the colloidal solute particles present in the plasma are responsible for a very minor portion of the freezing point depression effect.

** Probably a guitarfish, *Rhinobatos undulatus.*

samples from *Mustelus canis,* and from 870 mg.% to 1320 mg.% in samples from different species of *Raja* (Smith, 1929). The highest BUN level was 1380 mg.% reported for *Scyllium catulus* (von Schroeder, 1890). The blood's non-protein nitrogen (NPN) level has been measured by Denis (1913, 1922), Smith (1929), and Cohen *et al.* (1958). In *Carcharhinus obscurus, Carcharias littoralis* (= *C. taurus*), *Mustelus canis* and *Squalus acanthias* the NPN level ranged from 1000 to 1180 mg.%; all but about 130 to 190 mg.% was accounted for as urea. Trimethylamine oxide and betaine are probably responsible for most of the excess (Smith, 1936; Cohen *et al.,* 1958). Ammonia is present in very small amounts in elasmobranch blood. Smith (1929) was unable to detect any ammonia in the blood of *M. canis* or of *Raja diaphenes* (= *R. ocellata*) and only a trace in *C. littoralis.* Denis (1922) reported blood ammonia levels of 1.6 mg.% for *M. canis* and 1.7 mg.% for *C. obscurus.* The nutritional states of the animals could account for the two-fold differences (28 and 56 mg.%) in amino acid nitrogen levels reported for *M. canis* by Denis (1922) and Doolittle *et al.* (1960), respectively.

C. obscurus had 31 mg.% and *C. taurus* had 39 mg.% according to Denis (1922). The total protein content of elasmobranch plasma is also highly variable. Of 13 species which have been examined the lowest value was 1250 mg.% for the plasma of *Rhinobatos productus* (Urist, 1961). Reported values generally are in the range of 2800 to 3200 mg.% (Dill *et al.*, 1932; Para, 1936; Cohen *et al.*, 1958; Doolittle *et al.*, 1960; and Urist, 1961).

The relationships of the vascular compartment to the external environment, and the osmotic advantages of urea retention and elevated blood salt concentrations of elasmobranchs have been summarized by Smith (1936). But the relationships of the vascular compartment to other fluid-containing cavities within the elasmobranch body are not as well-known or understood. The aqueous humor has received attention (Kisch, 1930; Doolittle *et al.*, 1960; Stone and Dewell, 1962; Maren, 1962). So have the cranial cavity fluid (Hogben *et al.*, 1960; Murray and Potts, 1961; Maren, 1962) and the fluids of the labyrinth (Kisch, 1930; Murray and Potts, 1961). Pericardial fluid practically fills the rather rigid pericardial cavity and could influence cardiac dynamics. But observations upon the fluid are not numerous and hardly systematized. The same could be said of the rather voluminous perivisceral fluid. For a summary of available data, see Table III.

Table II

Average concentrations of some inorganic solutes in plasma of various elasmobranchs

Group	Species	Solute (mEq./L.)					Reference
		Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-*	
Selachians	*Aprionodon isodon*	238	7.0	—	—	252	Sulya *et al.* (1960)
	Carcharhinus limbatus	258	10.0	—	—	241	Sulya *et al.* (1960)
	Carcharias taurus	268	9.2	9.2	14.1	298	Macallum (1926)
	*Mustelus canis***	288	8.0	5.0	3.0	270	Doolittle *et al.* (1960)
	Scyliorhinus caniculus	303	—	8.5	—	303	Para (1936)
	Squalus acanthias	258	6.9	8.0	12.5	280	Macallum (1926)
	Squalus acanthias	286	5.7	5.2	—	246	Burger and Hess (1959)
	Squalus acanthias	263	4.1	6.6	—	249	Rodnan *et al.* (1962)
	Squalus acanthias	—	6.6	—	—	251	Hogben *et al.* (1960)
	Squalus acanthias	255	6.6	—	—	239	Maren (1962)
	Squalus acanthias	223	3.3	—	—	241	Robin *et al.* (1964)
	Sphyrna tiburo	289	12.5	—	—	245	Sulya *et al.* (1960)
Batoids	*Platyrhinoidis triseriata*	234	11.4	10.5	9.1	208	Urist (1961)
	Raja clavata	285	4.0	—	—	240	Enger (1964)
	Raja clavata	288	—	7.0	—	287	Para (1936)
	Raja clavata	289	4.0	—	—	311	Murray and Potts (1961)
	R. erinacea	260	3.9	—	—	253	Maren *et al.* (1963)
	R. laevis	182	4.2	—	—	220	Maren *et al.* (1963)
	R. ocellata	285	3.5	—	—	255	Maren *et al.* (1963)

* Scott (1913) reported 246 mEq. of Cl^- per liter for *M. canis* and Chaisson and Friedman (1933–34) a 260 mEq. of Cl^- per liter for *R. erinacea*.

** Compare with data presented in Table III.

Our objective was to measure the osmotic relations and the major solute com-

TABLE III

Comparison of average concentrations of some components of the blood, plasma or serum (S), pericardial fluid (PC) and perivisceral fluid (PV) of some sharks and skates. Numbers in boldface represent reported significant differences from plasma values.*

Species	Fluids	pH	Inorganic components (mEq./L.)						Reference
			Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	Urea–N mg.%	
Carcharias taurus	S	7.4	267	5.5	11.0	4.6	235	1060	Smith (1929)
	PV	**5.8**	276	8.9	10.2	**42.8**	306	1000	
	PC	**5.4**	290	**9.0**	**5.7**	5.4	—	—	
Squalus acanthias	S	7.5	263	4.1	6.6	3.1	249	1040	Rodnan *et al.* (1962)
	PV	**5.7**	**296**	4.4	**4.3**	**7.3**	**328**	1210	
Raja erinacea	S		254	8	12	5	255	960	Hartman *et al.* (1941)
	PV		(201–463)	10	(3–18)	42	300	—	
R. laevis	S	7.4	255	4.9	7.5	5.6	241	1270	Smith (1929)
	PV	**5.8**	284	5.8	5.0	**35.8**	309	1280	
	PC	**6.4**	335	**20.3**	**1.6**	5.0	370	**1010**	
R. ocellata	S	7.3	237	6.8	10.2	7.0	227	1050	Smith (1929)
	PV	**5.8**	156	6.2	6.4	—	188	**715**	

* Kisch (1930) reports all three fluids had about the same amount of urea in *Torpedo ocellata* (about 800 mg.%) and the plasma and pericardial fluids of *Trygon violacea* had equivalent amounts (1090 mg.%).

ponents of stingray perivisceral and pericardial fluids and relate these to the plasma constituents and to the environment. We have made a preliminary report on our 1963 collections (Wynn and Bernard, 1964). It should be recalled that in elasmobranchs in general the coelomic cavity is potentially in continuity with the environment *via* abdominal pores, and that the pericardial cavity is potentially in continuity with the peritoneal cavity *via* pericardioperitoneal canals in the transverse septum.

We wish to thank Professor E. Lowe Pierce, Director of the Sea Horse Key Marine Laboratory of the University of Florida, for making available the facilities of the laboratory, and to Mr. Bruce Campbell, Custodian of the Marine Laboratory, for his hospitality and able assistance in collecting the stingrays. We also wish to thank Doctors W. K. Hall and Margaret Coryell, Department of Biochemistry, Medical College of Georgia, for their analyses of the amino acids of the pericardial fluid.

MATERIALS AND METHODS

Animals were captured during the daylight hours at low water in the shallows off Sea Horse Key, Florida, or neighboring small islands of the Cedar Keys (29°8′N.; 83°2′E.). Periods of collection were: March 15–19, June 3–14, and July 22–30, 1963 and June 8–12, 1964. Stingrays were speared through a "wing" (pectoral fin) and then quickly hoisted aboard the skiff. Some rays had been pursued before capture. In 1963, 25 specimens of *Dasyatis americana* were captured; of the 11 specimens captured in 1964, four were of another species, *D. say.* In 1963 only one male was collected; 9 of the females were pregnant. In 1964, two males, both *D. say,* were collected; three females, all *D. americana,* were pregnant. The average width of all the *D. americana* specimens was 71 cm. (range: 41–92 cm.).

After inverting the animal the ventral surface was blotted dry. A U-shaped incision beginning and terminating at the coracoid bar was made. After reflecting the skin and hypobranchial musculature posteriorly, the pericardial sac was exposed and incised. A sterile, chemically clean eyedropper was used to collect the sample which was immediately placed in a sterile, previously unused, chemically clean, rubber-stoppered, 10-ml. tube (B-D Vacutainer). Because of the relatively large volume of fluid, sometimes two tubes had to be utilized. The perivisceral fluid was collected in the same way through a midline abdominal incision. Neither incision resulted in noticeable hemorrhage. While blood was being collected, the pH of aliquots of the pericardial and perivisceral fluids was measured, first with a Photovolt Model 180 pH meter and then with Hydrion narrow range pH papers (Micro Essential Laboratory, Brooklyn, New York). For blood collection an 18-gauge needle attached to a 10-ml. syringe was inserted into one of the posterior cardinal sinuses and the blood gently aspirated. Later we found that blood was more conveniently obtained by cardiac puncture. The blood sample was carefully transferred to a 15-ml. centrifuge tube. After 5 minutes centrifugation in a hand-powered centrifuge, the serum was separated, and its pH measured. The serum was then placed in a Vacutainer tube. Upon filling, all tubes were partially evacuated and placed in a dry-ice cabinet for quick freezing of the samples. The samples were kept frozen until other determinations were made. Total time elapsing between capture and fluid collection seldom exceeded 15 minutes. When fluid collection was completed the animals always had active cardiac movements and generally had active respiratory movements.

Total osmolality was determined cryoscopically with a Fiske Model H osmometer. Total protein was estimated by the method of Lowry *et al.* (1951); urea-N and NH_3-N, by a modification of the microdiffusion method of Conway (Hawk *et al.,* 1954); Na^+ and K^+, by flame spectrophotometry; Cl^-, by the method of Schales and Schales (1941); Ca^{++}, by the method of Bachra *et al.* (1958); and Mg^{++}, by the method of Andreasen (1957). Amino acid concentrations were determined by chromatographic separation on a Spinco Amino Acid Analyzer, Model 120B.

RESULTS

The results of the fluid analyses are summarized in Tables IV and V.

In comparison to the slightly acid serum, the pericardial and perivisceral fluids are considerably more acid. We also observed that the acidity diminished during the first 90 minutes after collection. As others have observed in other elasmobranch species (Table I), the stingray's blood is hyperosmotic to the seawater environment which, in the area where our specimens were collected, is itself highly variable osmotically. Our 1964 data indicate that the fluid contents of the two serous cavities have significantly fewer solute particles than the vascular

TABLE IV

Composition of D. americana and D. say body fluids. In the case of D. americana the data are expressed as mean values ± standard deviation and the number of observations is in parentheses below. Because only a few data were obtained for D. say (all collected in 1964), only the means are tabulated. Abbreviations as in Table III.

	Fluid	pH	mOsm./Kg.	Inorganic solutes (mEq./L.)					Organic solutes (mg.%)		
				Na^+	K^+	Ca^{++}	Mg^{++}	Cl^-	urea-N	NH_3-N	Total protein
D. americana (1963 data)	S	6.3±0.4 (2)	883±69 (18)	238±25 (20)	17.0±4.3 (18)	8.2±1.5 (13)	3.2±0.9 (8)	238±23 (10)	1064±158 (18)	4.4±2.5 (17)	4120±1520 (18)
	PV	5.3±0.1 (5)	849±91 (13)	239±22 (16)	14.0±2.0 (13)	3.1±1.2 (16)	0.7±0.2 (18)	260±27 (15)	1126±217 (13)	50±17 (13)	137±48 (11)
	PC	5.5±0.3 (8)	861±88 (19)	246±40 (25)	10.0±1.8 (21)	1.1±0.3 (20)	1.6±0.6 (14)	287±30 (23)	1020±204 (18)	36±12 (18)	41±21 (19)
D. americana (1964 data)	S	6.9±0.0 (4)	864±30 (6)	251±23 (6)	18.8±1.5 (6)	23.2±0.9 (6)	3.8±0.9 (6)	256±11 (6)	983±70 (6)	2.3±0.8 (6)	3550±700 (5)
	PV	5.4±0.1 (6)	827±23 (6)	255±12 (6)	20.4±1.1 (5)	5.1±1.1 (6)	0.6±0.1 (6)	310±31 (6)	1021±51 (6)	31±8.4 (6)	111±69 (6)
	PC	5.4±0.1 (6)	828±22 (6)	262±13 (6)	11.9±0.3 (6)	1.8±0.6 (6)	1.3±0.4 (6)	308±31 (6)	951±52 (6)	23±2.4 (6)	31±9 (6)
D. say	S	6.0	840	256	20.6	19.5	3.1	262	1069	2	2600
	PV	5.5	813	236	20.5	7.7	3.0	289	998	44	153
	PC	5.4	810	258	11.7	3.7	1.6	295	992	37	40
Sea water—1963		8.2±0.0 (2)	836±18 (3)	405±25 (3)	14.0±1.0 (3)	37.1 (1)	—	424±0.0 (2)	None detectable		
Sea water—1964		8.2±0.0 (2)	709±22 (6)	346±7.2 (6)	21.4±1.5 (6)	40.1±2.5 (6)	59±6.4 (6)	399±11 (6)	None detectable		

TABLE V

Probability values derived from comparison of body fluids of D. americana. A dash indicates no significant difference, i.e., P. >0.05*. Abbreviations as in Table III.*

Fluids	Year	pH	Osmolality	Na$^+$	K$^+$	Ca^{++}	Mg^{++}	Cl$^-$	Urea	NH$_3$-N	Total protein
S:PC	1963	<0.05	—	—	<0.01	<0.01	<0.01	<0.01	—	<0.01	<0.01
	1964		<0.05	—	<0.01	<0.01	<0.01	<0.05	—	<0.01	<0.01
S:PV	1963	<0.05	—	—	<0.05	<0.01	<0.01	<0.05	—	<0.01	<0.01
	1964		<0.05	—	—	<0.01	<0.01	<0.05	—	<0.01	<0.01
PC:PV	1963	—	—	—	<0.01	<0.01	<0.01	<0.05	—	<0.05	<0.01
	1964	—	—	—	<0.01	<0.05	<0.05	—	<0.05	—	<0.05

compartment. Although the 1963 data were similar, the variability was greater and the differences were not significant.

Within the stingray, both Na$^+$ and Cl$^-$ concentrations were less than environmental concentrations and apparently were independent of rather large shifts in the latter (*cf.* 1963 and 1964 sea-water data). There were no significant differences of sodium concentration between the three compartments. Chloride, on the other hand, was significantly elevated in the two serous cavities. The pericardial fluid contained significantly less potassium than either the serum or perivisceral fluid. The bivalent cations, magnesium and calcium, were present in significantly smaller amounts in the two serous fluids. The pericardial fluid contained significantly less calcium and significantly more magnesium than the perivisceral fluid.

Like sodium ions, urea molecules were in equilibrium between the blood and the two serous cavities; ammonia, on the other hand, was much more concentrated in the perivisceral fluid and only slightly less so in the pericardial fluid. Corresponding to the elevated Cl$^-$, there was very little protein in the two serous fluids.

Fluids obtained from *D. say* were (with the exception of Mg^{++} concentration in the perivisceral fluid) chemically similar to those from *D. americana.*

Eleven amino acids were detected in a sample of pericardial fluid from *D. americana.* Their concentrations (in micromoles per cent) were: aspartic acid (0.71), threonine (0.52), serine (2.77), asparagine plus glutamine (0.76), glycine (3.00), alanine (1.16), isoleucine (0.40), leucine (0.97), ornithine (1.09), and taurine (3.09). In addition there was noted a "possible trace" of arginine, and two unknown ninhydrin-positive compounds present in relatively large amounts (3.93 and 2.77 micromoles per cent).

DISCUSSION

The relative acidity of the pericardial and perivisceral fluids of several species had been observed by Smith (1929). Rodnan *et al.* (1962) made the same observation upon the perivisceral fluid of *Squalus* (see Table III). Our generally lower values might reflect species differences, or collection techniques which provided fluid samples under optimum field conditions.

As noted above, elasmobranch blood osmolality is highly variable and related to the environmental osmolality. Our observations would indicate that the relationship may not be too rigorously regulated. Osmolar differentials exist between the three fluid compartments, with the blood hyperosmotic to the serous fluids.

Since there have been no comparable studies on other stingray species, comparisons of *D. americana* or *D. say* solute concentration data with data reported for remotely-related species would have little value. In general, our data are not notably different from those reported by others. There are two noteworthy exceptions: Rodnan *et al.* (1962) reported significantly more sodium in the perivisceral fluid of *Squalus*. We found no difference. Secondly, Campbell (1961), using a different analytical technique, reported that the plasma of the closely related species, *D. centrura,* contains up to 32 micromoles per cent of arginine. This is about ten times the concentration of any amino acid the amino acid analyzer was able to detect. In fact, we found only a "trace" of arginine. Arginine present in our samples originally might have been lost through long storage.

The data, as well as those reported elsewhere in the literature, clearly indicate that artificial physiological bathing fluids simulating plasma may not contain optimal ion concentrations for all elasmobranch viscera. In studies of elasmobranch heart physiology, for example, not only would one have to consider the almost rigid musculo-skeletal ensheathment of the pericardium (Lyon, 1926; Dohrn and Rein, 1949–50) and the apparently normal, and perhaps physiologically necessary, cardiac tamponade but also the factors of high H^+ and low K^+, Mg^{++} and Ca^{++} concentrations in the synthesis of any pericardial fluid substitute.

That the abdominal pores in these species of stingray probably do not permit entrance of sea water can be inferred from the gross ionic dissimilarities between sea water and perivisceral fluid. (Of course, special ion "pumps" might be present.) Similarly, mechanical exchange of fluids through the pericardioperitoneal canals must be limited. Because of the tamponade and respiratory movements, passage from the pericardial cavity to the abdominal cavity is more likely than flow in the opposite direction.

SUMMARY

1. The pH, osmolarity, Na^+, K^+, Ca^{++}, Mg^{++}, Cl^-, urea, NH_3 and total protein content of the serum (S), pericardial fluid (PC) and perivisceral fluid (PV) of the stingray, *Dasyatis americana,* were estimated and compared to environmental data.

2. The PC and PV were found to be more acid and osmotically less concentrated than S. Although there were no significant differences in either Na^+ or urea concentrations in the three body fluids, K^+ was significantly reduced in PC. Ca^{++} and Mg^{++} were significantly less concentrated in PC and PV. Both PV and PC had elevated Cl^- and almost no protein; both contained significantly elevated NH_3 concentration. It follows that recipes for synthetic bathing fluids for elasmobranch viscera should not be based upon data obtained from plasma or serum analyses.

3. A few specimens of *D. say* were collected. The data were similar.

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A NEW APPROACH TO THE PROBLEM OF AGGREGATION IN THE CELLULAR SLIME MOLDS[1]

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The prime reason for initiating this project was a conviction that we will never begin to understand the mechanism of aggregation in the cellular slime molds and the subsequent developmental stages unless we know the chemical identity of acrasin, the chemotactic agent responsible for the attracting of the amobae to central collection points. With this object in mind a new assay was sought for acrasin which would be serviceable for a chemical analysis. As will be evident from what follows, a practical bio-assay has been developed, but we cannot be certain until the substance or substances we are testing for are completely isolated and known, whether or not we are without question testing for acrasin and not some associated substance. Either result would be a significant advance in our knowledge of slime mold development, but by the very nature of this dilemma, this paper is a progress report rather than the final analysis of a complex problem.

The details shall be presented shortly, but briefly the matter was approached by preparing stable solutions of acrasin by previously known methods primarily devised by Shaffer (1956a, 1956b). It was noticed in the presence of these solutions the amoebae moved more rapidly and furthermore this could be turned into a quantitative test, for the speed of the amoebae is proportional to the concentration of the extracted preparation. The question arises as to whether the preparation contains both an orientation factor (acrasin) and a separate rate-increasing factor, or whether acrasin does both. Despite preliminary chemical purification, we have been unable to separate the two components. Should acrasin have both of these biological effects (a matter which can only be settled when the chemical analysis now in progress is finished) then, as we shall see presently, our traditional assumptions as to how aggregation is initiated will have to be radically altered.

Before presenting this work, a short survey of previous attempts to identify acrasin should be given. The first assay was devised by Shaffer (1953, 1956b) which consisted of sandwiching amoebae, which are in the process of aggregating, between glass and a small block of agar, and adding the test solution to the meniscus. If it is active the amoebae will orient and move towards the meniscus. By this test Shaffer was able to prove conclusively that acrasin was a substance and that cell-free solutions could attract amoebae. Furthermore he showed that it was dialyzable (confirming the previous evidence of Runyon, 1942), that it was heat-

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stable, and that it was destroyed, presumably enzymatically, in the presence of live slime mold cells (which was confirmed by Sussman, Lee and Kerr, 1956).

Partly because the test is difficult to perform, and partly because it is not a quantitative assay, its use as a tool for chemical analysis is limited. Despite these shortcomings the test has been used in a number of attempts to identify acrasin: Sussman, Lee and Kerr (1956) reported two fractions which needed to be mixed in specific proportions to obtain activity; Sussman, Sussman and Fu (1958) subsequently added a third factor to the complex which also showed some activity when used alone. Wright and Anderson (1958) gave evidence that acrasin was a steroid, and Heftmann, Wright and Liddell (1959, 1960) found stigmastenol to be present in slime molds. However, doubts still remain concerning the chemical nature of acrasin and these were reinforced by the work of Hostak and Raper (1960) who showed, using another technique of Shaffer, that various alkaloids had some sort of stimulating effect on the aggregation of *Acytostelium.*

Recently Francis (1965) has devised a most interesting new quantitative assay involving the flowing of test solutions over cells aggregating under a layer of gently moving standard salt solution. The full potentialities of this test have only begun to be realized and Francis has already made some worthwhile observations on the mechanism of aggregation. The only difficulty with the assay is that it is technically delicate and demanding, a drawback it shares with the Shaffer test.

THE ASSAY

The object of the assay is to record the speed of the amoebae bathed in the test solution. Originally this was done by following individual amoebae with a compound microscope and making camera lucida drawings at regular intervals. While this is perfectly satisfactory it was found tedious and impossible for one person to test more than one solution at a time.

To circumvent these difficulties a standard procedure was devised so that eight or ten solutions could be tested simultaneously. The method takes advantage of the fact noted by Samuel (1961) that the amoebae tend to repel one another. Therefore if they are placed in contact with the test substance in a concentrated group they will spread, the individual amoebae going in fairly straight lines away from the dense center. If their progress is measured at two instants in time, the average rate of outward movement may be easily computed.

The precise method is as follows: 1 ml. of the test solution is carefully mixed with 1 ml. of hot 4% Bacto agar (on a slide-warming plate kept at 60° C.) in a small plastic petri dish (50 × 12 mm.—Falcon No. 1006). After the agar has solidified the covers are removed from the dishes for 10 minutes to allow excess moisture on the surface to evaporate. The amount of surface moisture has a great effect on the rate of movement, as Samuel (1961) showed, and every effort to keep standard moisture conditions is desirable. Since with each set of experiments one petri dish is run as a plain agar control, it is possible to some extent to take into account these fluctuations in the humidity of the environment.

The test amoebae may be prepared by any method which produces a suspension of centrifuge-washed cells. All the liquid culture methods are entirely satisfactory. We used the method described previously (Bonner, 1947) in which the amoebae are grown with the bacterium on a buffered 1% peptone and a 1% dextrose agar over which 2 or 3 ml. of sterile distilled water had been poured. Two of such plates are incubated at 22° C. for 48 hours and then washed free by two two-minute centrifugations at low gravitational force. The final amoebae are in a dense suspension in standard salt solution (Bonner, 1947).

These amoebae are allowed to settle on small squares of uncoated cellophane, roughly 16 mm^2. (Visking dialyzing membrane), which are placed on the bottom of a petri dish filled with standard salt solution. After 20 to 30 minutes settling time the squares are removed with forceps and each dried by placing it on a piece of filter paper and then taking a corner of another piece of filter paper and touching the square in the center to remove any remaining water. The square is then placed on one of the test petri dishes, amoeba side up, three such squares being placed in each dish.

After about one hour it is possible to see that the amoebae have crossed the edge of the cellophane onto the agar. With a pair of dividers 6 grooves 2 mm. in width are scratched on the bottom of the plastic dish perpendicular to 6 sides of the cellophane squares. Then with the use of an ocular micrometer in a dissecting microscope the distances between the edge of the cellophane and the four cells farthest out are measured and averaged for each of the 6 grooves. The same procedure is repeated after two or four hours and in this way it is possible to have an index of the rate of outward movements of cells which can be expressed in terms of mm./hr. It should be pointed out that one of the advantages of the cellophane square is that it provides sharp starting lines for the spreading amoebae (Fig. 1A).

In order to give some idea of the consistency of this test, four different sets of data have been chosen at random from our notebook and presented in Table I. For each there is one control and one experimental. As a convenient rule of thumb it was considered doubtful that the difference between experiments and controls was significant if their ranges overlapped at all. It is not obvious from the small amount of data in the table what might be the advantages of the four- as compared to the two-hour reading. If a comparison is made of the size of the ranges of one-, two,- and four-hour measured intervals it was found that the mean range was 0.17 for one-hour intervals, 0.09 for two-hour intervals and 0.07 for four-hour intervals. In other words the four-hour interval may be more precise than the two-hour, but if the difference between the experimental and control is marked, this extra precision is hardly necessary.

One of the first questions that arose was to what extent the result was affected by the age of the amoebae, for unless some accurate method is used such as the Gerisch (1959, 1960) liquid culture method, the physiological age of the amoebae we use must vary considerably. In 5 experiments 24-hour amoebae were compared with 48-hour amoebae and there was no significant difference in the result. In some cases the amoebae were clearly very advanced and began aggregation immediately, yet those cells that escaped from the cellophane squares did so at the same rate as younger cells. All the tests so far indicate that test cells of any age give similar results on the same test agar.

Another variable examined was cell density but again the result was identical, regardless of the density of the cells on the cellophane block. If the cells were too

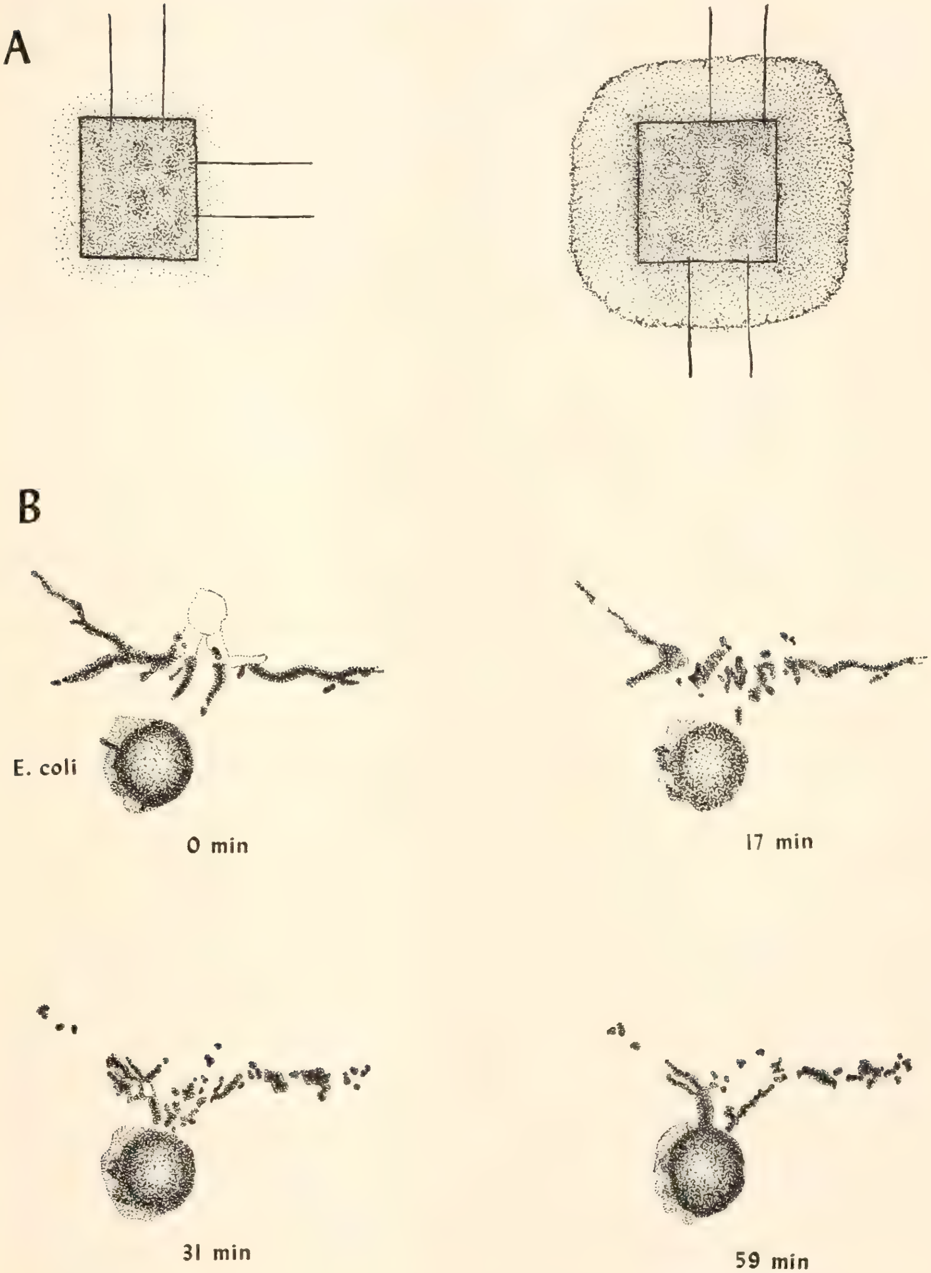

FIGURE 1. A. Drawings of two photographs of the assay. The control is at the left, and an experimental with active rate substance is shown on the right. The central cellophane square is about 4 mm. long. B. Drawings of a series of photographs showing the effect of a colony of *E. coli* on aggregating amoebae. In the first illustration (0 min.) the outline of the position of the original center before removal is indicated. The width of the *E. coli* colony is about 300 μ.

TABLE I

Four experiments and their controls picked at random. The first two are measured over a 4-hour period and the second two over a 2-hour period. Each column of 6 figures represents the 6 marked sides of the 3 cellophane squares on one petri dish. Each figure is the mean of the 4 fastest amoebae at about 1 hour subtracted from the 4 fastest at 2 (or 4) hours later. Since the controls vary somewhat (possibly due to moisture conditions) the experimental figure can be considered in terms of per cent increase over the control. The standard deviations are of the 6 means (of 4 amoebae each). This is equivalent to the standard error of the average of all the 24 amoebae involved in each test.

Date	Control in mm./hr.	Mean	Standard deviation	Experimental in mm./hr.	Mean	Standard deviation	% Increase of experimental
9 April '65	0.33			0.71			
	0.37			0.79			
	0.34			0.71			
	0.30			0.66			
	0.36			0.71			
	0.31	0.34	0.03	0.70	0.71	0.04	109%
13 April '65	0.35			0.78			
	0.31			0.70			
	0.42			0.87			
	0.36			0.86			
	0.30			0.72			
	0.42	0.36	0.05	0.76	0.78	0.07	117%
26 May '65	0.30			0.92			
	0.32			0.95			
	0.23			0.94			
	0.25			0.73			
	0.21			0.90			
	0.23	0.26	0.04	0.90	0.89	0.08	242%
31 May '65	0.35			0.68			
	0.29			0.58			
	0.30			0.68			
	0.37			0.63			
	0.41			0.49			
	0.38	0.35	0.05	0.56	0.60	0.08	71%

few the only disadvantage was that it was difficult to find enough cells to measure, but should they be found, their rate of outward movement was the same as with dense populations, all other conditions being equal. In general we found that fairly concentrated amoeba densities were the most convenient. We attempted roughly to cover the cellophane with a solid layer of cells, but we knew that any variation did not affect the test results.

Finally some experiments were run to see if any of the more obvious solutes in the test agar might affect the rate of movement. In agreement with the results of Samuel (1961), phosphate buffer, standard salt solution, as well as non-toxic concentrations of NaCl had no effect on the rate of movement. Also 0.1% peptone was tried in the test agar, again with negative results.

Quantitative nature of the assay

The first indication that the test was quantitative came with some early experiments with single amoebae. If the concentration of the test solution was diluted, the speed was apparently reduced. To determine the exact relation a series of 1/5 dilutions over a 25-fold range were run using the cellophane square assay method and in 9 separate experiments it was shown that a 5-fold increase in concentration produces a 1.2-fold increase in the rate of movement. It is not known at present over how great a range of concentration this relation holds, although when the chemical work is at a more advanced stage this question will be pursued further. Under ideal conditions the plain agar controls run about 0.30 mm./hr., and with very active solutions it is possible to obtain speeds over 1.00 mm./hr. If the rate/concentration ratio holds over this range, then if we arbitrarily give a rate of 0.39 mm./hr. a concentration of 1, then 1.00 mm./hr. should indicate a concentration between 10^3 and 10^4 times greater.

Shaffer tests

Throughout the experiments to be described periodic Shaffer tests were performed, following the procedure outlined by Shaffer (1956b). For any one test solution the tests were run numerous times to make certain of the result. Even a positive result must be interpreted with caution for it is always possible that new centers arise near the edge which could attract the amoebae. To give an idea of the type of results obtained, one solution was tested in 19 separate Shaffer tests. In 8 tests the result was clearly positive; in 11 tests the result was doubtful or clearly negative where there was no hint of orientation towards the edge of the agar block. From this experiment it was concluded that the test solution did contain an orienting, chemotactic factor. The variation in the results is presumably due to differences in the conditions of the amoebae (which must be in the process of aggregating before they are placed under the block) and to the skill of the operator in the handling of the agar blocks.

RESULTS

Methods of obtaining active solutions

The original solutions which were used to perfect the test were prepared by a method devised by Francis (1965), based upon the original technique of Shaffer (1956a, 1956b). It consists of washing cells by centrifugation and allowing them to settle on the bottom of a glass dish containing distilled water. As soon as aggregation begins the water is changed for fresh distilled water, which, after 30 minutes over the aggregating amoebae, is removed and as quickly as possible brought to a boil. In these early experiments and the majority of all subsequent experiments we concentrated this original solution 200 times by boiling. This was usually done by bringing the preparation to dryness and then redissolving the residue in enough water to give a 200 × concentration.

Once the test was devised, immediately attempts were made to improve the method of obtaining more active solutions. We will not discuss here (for the work is still in progress) the chemical methods of purification and concentration, but rather the basic method of obtaining active solutions from aggregating amoebae. But before we proceed any further it may be wise to give a provisional name to the active substance. We cannot call it acrasin since that substance is defined as being involved in aggregation chemotaxis and here we are testing for rate of movement.

Even though the preparations have been tested at intervals by the Shaffer test with positive results, we cannot be sure as yet that there are not two or more substances. It is even conceivable that there is more than one orienting substance but all of these matters must await the results of the chemical analysis before they can be settled. Since here we are testing primarily for the substance which increases the rate of movement of the amoebae let us for the time being call it the *rate substance,* and leave in abeyance the question of whether or not this is the same substance as acrasin.

In this series of experiments the amoebae that were used to produce the rate substance were prepared by the liquid nutrient medium devised by Sussman (1961). The amoebae were fed with *E. coli* B/r and shaken for 48 hours at 22° C. In order to reduce the labor, the amoebae were somtimes suspended in Pyrex baking dishes (35 × 22 cm.) in distilled water about a centimeter deep, although these gave less consistently satisfactory results than the petri dishes, presumably because the amoeba density was more difficult to control. (The reason for not giving exact data on amoeba density will soon be clear; better methods of obtaining the rate substance have been devised.)

One of the difficulties was that there often was a considerable precipitate after boiling. To circumvent this the amoebae were placed in dialyzing bags with distilled water and these were placed in a Pyrex baking dish filled with distilled water. If these were set up in the evening, by next morning it could be seen that aggregation had occurred within the bags. The water outside the bags was poured off into a beaker and boiled down 200×. It gave a clear and precipitate-free solution, and although it showed considerable rate substance activity, it had roughly the same potency as the solution prepared without the dialysis bags and concentrated 200×. The controls gave an average of 0.37 mm./hr. (four cases); two cases in which the bags were gently shaking averaged 0.48 mm./hr., and two cases in which the dishes were still averaged 0.55 mm./hr. Note that there is a slight advantage when the preparation is not agitated.

As there was no appreciable gain from the method involving dialysis bags, the simpler method involving the decanting of the water from over the amoebae was resumed. It was found that the precipitate could be effectively removed by filtering and we were able to show that one could filter either after the solution was first brought to a boil or after it had been boiled down; the activity was about the same for both methods.

Two experiments were performed to test the possibility that some of the potency of the solutions was lost in boiling. Instead of boiling the solution it was concentrated 40× in a flash evaporator which takes off the water in a vacuum at 40° C. In two experiments the controls averaged 0.35 mm./hr., the vacuum evaporated 0.34 mm./hr., and a further aliquot reduced 40× by boiling gave a mean value of 0.48 mm./hr.

It is clear from this experiment and all the others reported in this section that the original boiling method of isolation is the simplest and most effective. The fact that all the activity was lost during the flash evaporation at 40° C. suggests that, similar to the observations of Shaffer (1956a, 1956b) and Sussman, Lee and Kerr (1956), an enzyme has been retained which specifically destroys the rate substance. Unfortunately whether or not this is the correct interpretation is still in doubt despite a number of experiments that have been run to clarify this point. Again it is hoped that when the chemical nature of the rate substance is known this matter will be settled.

Species specificity

A series of experiments were run involving *D. discoideum* (Strain No. 1), *D. mucoroides* (Strain No. 11) and *Polysphondylium violaceum* (Strain No. 6). Using our rate test it was impossible to show any species specificity. The rate substance prepared from *D. mucoroides* stimulated the amoebae of *D. discoideum* and *vice versa;* the rate substance of one was indistinguishable from the other in its effects. The only discernible difference was that *D. mucoroides* provided poor test amoebae (as did *P. violaceum*) in that the amoebae tended to aggregate too quickly and, especially in the controls, few amoebae escaped from the cellophane squares (Table II).

The same result was observed between *D. discoideum* and *P. violaceum*. *P. violaceum* rate substance was tried numerous times on *D. discoideum* test amoebae and again its effect equalled that of the *D. discoideum* rate substance.

This lack of genus specificity was surprising, in the light of the observations of Shaffer (1957a) who has presented good evidence that there is a separate *Polysphondylium* and *Dictyostelium* acrasin, although the former species produces both. However, Shaffer does point out that it is possible to interpret his result on the basis of one substance if one makes certain assumptions concerning a differential response to it by the two species.

To examine this question further we ran 10 Shaffer tests with our 200× concentration rate substance preparations of *P. violaceum* on *D. discoideum* test

TABLE II

The effect of the rate substance of different species upon one another (figures expressed in mm./hr.)

	D. discoideum test amoebae		*P. violaceum* test amoebae	
	Controls	Experimentals	Controls	Experimentals
D. discoideum rate substance	0.37	0.61 0.55	0.16	0.79 0.73
D. mucoroides rate substance	0.32	0.40		
P. violaceum rate substance	0.30	0.48	0 (aggregated)	0.45
	0.26	0.48	—	0.40
	—	0.50		
	—	0.49		
	—	0.45		
	—	0.40		
	—	0.51		
	0.34	0.40		

amoebae. In 6 of the tests the result was positive and there was strong orientation towards the meniscus, while the remaining four cases were doubtful or negative. There is, therefore, good evidence that a concentrate of water which has been sitting over *Polysphondylium* amoebae contains both a rate-increasing and an orientation factor for *Dictyostelium* amoebae. However, this completely evades the question of species specificity which clearly exists; it is a matter which will require much further work.

Effect of the rate substance preparations on the appearance of the amoebae

During the course of the assays a number of striking features in the appearance of the amoebae on the dishes containing the rate substance preparations were observed that were absent in the controls. It must again be cautioned, however, that we cannot at this time be certain that these effects are produced by the rate substance itself, but it is possible that they may be produced by some associated substance.

If the preparation is effective, and the rate higher than about 0.70 mm./hr., then there is often a sharp advancing edge in the experimentals that is totally absent in the controls (Fig. 1A). This resembles the feeding edge observed by numerous workers although here, of course, there are no bacteria and the cells are not feeding.

If, in a similar test with an active rate substance preparation, the test amoebae are about to aggregate, then another property is sometimes evident. The cells at the outer edge tend to adhere to one another in groups. Even more remarkable, on rare occasions the cells actually form streams which lead outward to the advancing edge. These streams never form centers and eventually they dissolve. Again, neither the streams nor the adhesive properties of the cells are seen in the controls.

It is of interest to note here that a similar pattern of streams without centers radiating away from a central point may be seen in the disintegration of the primary centers, and the formation of secondary centers in *D. minutum* as described by Gerisch (1964).

Effect of rate substance preparation on center formation.

The process of center formation and the inhibition or stimulation of centers is one that recently has received considerable attention (Bonner and Dodd, 1962; Bonner and Hoffman, 1963; Shaffer, 1963; Hohl and Raper, 1964; Gerisch, 1964; Kahn, 1964). Since centers will form directly upon the cellophane squares, and since the squares for the controls and the experimentals are prepared in the same way, it is possible to test whether or not the rate substance preparations will affect the number of centers per unit area (or the aggregation territory size).

It is very obvious that the test preparations do not affect the number of centers on the cellophane squares; the experimentals always resemble the control regardless of how effective the preparation is in increasing the rate of movement of the amoebae.

When is the rate substance produced and what kind of cells produce it?

These experiments began with asking the question of what periods, before, during and after aggregation, were the most active in producing the rate substance.

The amoebae after 48 hours incubation were washed by centrifugation and placed on the bottom of the Pyrex baking dishes under a layer of distilled water. This was done at 5 PM and the first collection of water to be concentrated was taken after three hours. During this time no aggregation had occurred. Fresh distilled water was added immediately over the amoebae and they were allowed to stand overnight (13 hours). This water was removed and concentrated, again adding fresh water to the dishes. One or two three-hour samples were collected the same way in the morning and each one was concentrated 200 ×. During this period aggregation had been completed and small centers were evident.

When these samples were tested the controls gave an average of 0.29 mm./hr. (four cases); the first three hours an average of 0.74 mm./hr. (four cases); the next 13 hours, 0.51 mm./hr. (three cases); the next three hours, 0.53 mm./hr. (two cases); and the final three hours, 0.53 mm./hr. (one case). It was not surprising to find that the stages after aggregation showed activity for it is well known that these stages produce acrasin, but it was most unexpected that the rate substance should be produced long before aggregation begins. The fact that the first reading was higher than the others is possibly not significant for it might contain more cells. When the cells are placed in distilled water they do not adhere well to the glass bottom and as a result many are lost with the first decanting.

To control this factor a series of five baking dishes were set out, care being taken that they contained exactly the same number of amoebae as well as the same amount of water. These were allowed to remain different periods of time, and each was poured off and boiled down exactly 200 ×. The entire experiment was run twice. The results of each were consistent and therefore they have been averaged. The controls gave 0.35 mm./hr. The 5-minute sample was 0.79 mm./hr.; the 15-minute sample 0.70 mm./hr., the 30-minute sample 0.69 mm./hr., the 120-minute sample 0.67 mm./hr., and the overnight (19-hour) sample 0.52 mm./hr.

The surprising result here is that in 5 minutes, again long before aggregation, more of the rate substance is given off into the water than any of the longer periods. There is, in fact, a clear decline in activity if the water is left standing over the amoebae for 19 hours.

The next step was to test the supernatant right in the testtube, directly following the second centrifugation of the amoeba-washing process. This was compared to the whole contents of the testtube (cells and supernatant) as well as the cells alone. It should be noted here that the absolute values have little meaning, for the amount of concentration is much less here since the original volume of water is less. Controls averaged 0.40 mm./hr. (three cases); the cell-free supernatant averaged 0.55 mm./hr. (two cases); the cells and the supernatant combined gave a value of 0.75 mm./hr. (two cases); and the cells alone were 0.68 mm./hr. (two cases). In one experiment the cells were sonicated and the resulting cell-free preparation gave a value of 0.76 mm./hr. It is clear from this that the cells themselves, long before aggregation, not only are continually giving off the rate substance, but the substance is contained within the cells also.

As an additional control, a suspension of *E. coli* was prepared, boiled down, and tested with the astonishing result of very high activity. Not only *E. coli*, strain B/r, but other strains which had been stored dry or frozen. In 9 experiments, lumping all the different kinds of *E. coli* preparations, the control values were 0.35 mm./hr. while the *E. coli* values were 0.94 mm./hr. These gave greater activity than had hitherto been observed. In many of the tests the exact concentration was unknown although a 5% preparation made with dry *E. coli* was especially effective. The highest value obtained was a rate of movement of 1.22 mm./hr., when the control ran 0.23 mm./hr. This is a 430% increase in the velocity of the cells.

The substance given off by the *E. coli* is also dialyzable and in fact has all the properties of the amoeba rate substance. The morphology of the cells under the influence of the rate substance derived from *E. coli* is indistinguishable from that of the amoebae. The *E. coli* rate substance was tested with the Shaffer test and in 19 tests, 8 were positive and 11 doubtful or negative, indicating without question that the *E. coli* rate substance possessed the ability to orient aggregating amoebae.

The final and most elegant demonstration of this fact can be readily made with a simple and direct experiment. Amoebae of *D. discoideum* were grown on non-nutrient agar, over which had been spread a thin layer of *E. coli,* the standard method of preparing the cells for the Shaffer test. When aggregation was in full progress, the center of an aggregate was removed by a hair loop and a small drop of fresh *E. coli* cells was placed about 100 μ from the side of a large stream. Within 15 minutes, the cells of the stream will break up and by 30 minutes they will form chains of cells leading into the colony of *E. coli* as though it were a center (Fig. 1). If the removed center is placed in a similar spot for comparison, it is clear that the normal slime mold center is stronger in orienting the aggregating cells and the cells respond more rapidly. But the fact that the streams of aggregating amoebae will form into colonies of *E. coli* is absolutely clear-cut.

Some experiments were run with yeast extract and it was found that this also contained a rate-increasing factor although in general it was less satisfactory as a source for the rate substance in that it apparently contained substances that were toxic to the amoebae as well. In three experiments the average of the controls was 0.35 mm./hr. while 1% yeast extract gave an average value of 0.65 mm./hr. It should be pointed out that since we can now obtain our rate substance from bacteria and yeast, the chemical analysis will be greatly facilitated; quantity is no longer a problem.

Evidence that the repulsion between cells does not involve the rate substance

As has already been discussed, if different concentrations of amoebae are placed on the cellophane square in the assay, there is no effect on the rate of movement of the cells. This is the first indication that the substance which is responsible for the repulsion between the amoebae is distinct from the rate substance. To test this possibility further, small blocks of agar containing a high concentration of the rate substance (*ca.* 0.70 mm./hr.) were placed different distances from an advancing edge of amoebae coming off a cellophane square in a control test petri dish. In no case was the advancing edge stopped by the presence of the rate substance in the block. In fact, quite to the contrary, the cells on that side of the cellophane square were stimulated in their rate of movement and passed right over the block, spreading away from the cellophane square at an increased pace. The evidence supports the notion that the mechanism of the repulsion between cells is totally independent of the rate substance.

DISCUSSION

We have at the moment five recognizable phenomena that have the appearance of being related. This appearance, however, could be quite misleading and we cannot know their relation until the substances involved have been isolated and identified.

The five phenomena are: (1) the orientation of amoebae in normal aggregation; (2) the orientation of amoebae to test substances in the Shaffer test; (3) the orientation of the vegetative or pre-aggregation amoebae to colonies of *E. coli;* (4) the orientation of aggregating amoebae towards colonies of *E. coli;* (5) the increase in rate of the amoebae at various stages. There are undoubtedly other phenomena as well, involving both rate and orientation of which we are still ignorant.

One extreme in the interpretation of the results accumulated thus far is that each of these phenomena is mediated by a separate substance; in fact they could conceivably each involve more than one.

The other extreme, and one which obviously has more appeal, is that they are all mediated by one substance. As has been said before, this matter is currently under investigation (with the generous help and advice of Dr. A. B. Pardee and his associates), but it may still be some while before it is resolved. In the interim let us see what are the present arguments to favor such an hypothesis and, if it is assumed to be the case, what are the consequences as far as slime mold development is concerned.

The preparations of rate substance have the property of inducing adhesiveness and stream formation, a property characteristic of acrasin, as Shaffer (1957b) showed. This has the flaw that many substances might have this property, or that the acrasin has not been separated from the rate substance.

The idea that rate increase and orientation might go hand in hand is consistent with one old observation. It has long been known from time-lapse films of aggregation that the cells often move inward in waves or pulses that start in the center and radiate outward. It has been assumed that these are pulses of acrasin secretion, and the pertinent point is that the outgoing waves are characterized by periods of fast inward movement of amoebae. In other words if the pulses are indeed periodic puffs of acrasin, these puffs simultaneously accelerate and orient the amoebae.

Another observation made from an old film would also fit in with the idea that rate and orientation go together. When a center is removed during aggregation the streams break up, but they continue to pulse; the cells repel one another but do so in definite intervals of fast movement. If, as it has always been assumed, the pulses are associated with acrasin secretion, then acrasin continues to be emitted in puffs, yet there is no centripetal acrasin orientation.

This paradox is eliminated if we consider aggregation to be characterized by a period of especially high orientation sensitivity to acrasin, so high in fact that it overrules the separate cell repulsion phenomenon. According to this hypothesis, then, the removal of the center caused a momentary loss of orientation sensitivity on the part of the cells, not a loss in acrasin secretion, the existence of which is demonstrated by the pulses of fast movement.

This would also explain why the Shaffer test only works with amoebae that are in the process of aggregation, and not cells just about to aggregate. One must have cells which are in this state of high sensitivity.

The important point is that if we assume all orientation and rate-increasing to be achieved by one substance, then our traditional picture of the mechanism of aggregation must be radically altered. We can no longer think of the onset of aggregation as the moment when cells begin both to secrete and become sensitive to acrasin. Acrasin may be present very early, even in the food source, and is presumably a means of the amoebae reaching the food. There is a weak ability to orient the amoebae at this stage, as Samuel (1961) showed, but the separate cell repulsion phenomenon keeps the cells to some degree dispersed. After the food supply is depleted something occurs rather suddenly within the amoebae that produces a great increase in orientation sensitivity. As a result a single chemotactic factor can be used in the first instance as a means of finding food, and in the second as a means of aggregating and forming a communal spore-bearing structure.

Along with cell repulsion, it is clear that center formation and center inhibition are distinct processes from the orientation-rate factors described above. Besides these separate problems there are a number of others that need thorough investigation. For instance, there is the whole matter of how gradients of acrasin arise. This may be related to the interesting observations of Samuel (1961) who showed that the amoebae had different rates during different periods of their development, the period just prior to aggregation being one of especially slow movement. Another matter that needs further investigation is that of species specificity. Furthermore, the whole question of the repulsions (Bonner and Dodd, 1962) and attractions (Shaffer, 1964) of pseudoplasmodia in the air is puzzling and there is no information as to whether or not it relates in any way to the rate substance described here. Another point that should receive attention is that of the rate movement of the slugs. There have been conflicting opinions as to why larger slugs move faster than smaller ones (Bonner and Eldredge, 1945; Bonner, Koontz and Paton, 1953; Francis, 1962; Shaffer, 1962, 1964, 1965), and now we can suggest the further hypothesis that the larger slugs simply contain more of the rate substance.

Even if it turns out that the rate substance is utterly distinct from the orienting acrasin, its significance will be considerable, not only as to its role in slime mold development, but its role in the physiology of amoeboid movement. There are many new avenues of investigation that have been opened up by this approach.

SUMMARY

1. It has been shown that there is a substance (or substances) given off by the cellular slime mold amoebae that both orients aggregating cells (*e.g.* in the Shaffer test) and increases the rate of movement of the cells. A simple, quantitative assay has been developed to test the rate of cell movement. Using both types of test in conjunction, we have been able to show that the substance(s) is produced

before the aggregation of the amoebae and is even present in large amounts in the food source, *E. coli.*

2. The substance(s) is not species-specific; it induces adhesiveness in cells approaching aggregation; it does not affect center formation or center inhibition (territory size); nor is it related to the phenomenon of cell repulsion.

3. It is quite possible that there are many substances involved and the rate-increasing substance(s) is separate from orientation substance(s). Another possibility is that they are all one substance, which has hitherto been called acrasin. If this is the case then aggregation can no longer be considered the moment of first acrasin secretion, but one would have to assume that it is the moment of first acute sensitivity to acrasin, a sensitivity sufficient to override the cell repulsion phenomenon. It is hoped that if the chemical identification of the rate substance is achieved, then many of these important questions will be answered.

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NEUROCHEMICAL CONTROL OF GAMETE RELEASE IN STARFISH[1]

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The physiological factor(s) controlling gamete release in starfish had been largely ignored until 1959 when Chaet and McConnaughy first implicated the nervous system by extracting from radial nerves of starfish a hormonal catalyst promoting gamete shedding. Such neural extracts were capable of inducing the release of eggs or sperm when injected into mature starfish of the same species. Since then evidence has been steadily accumulating which suggests that the release of gametes from these echinoderms is, in part at least, controlled by a neurohormone found in their radial nerves.

Materials and Methods

Asterias forbesi from the American Atlantic coast, as well as two species of starfish from the Pacific coast, *Patiria miniata* and *Pisaster giganteus,* were used in the majority of these studies. Large donor starfish were collected for isolating nerves from which extracts were prepared containing gamete-shedding substance. The animals were obtained either by dredging or SCUBA diving and ranged in size from 15 to 20 cm. for *Asterias forbesi,* 7.5 to 15 cm. for *Patiria miniata,* and 20 to 25 cm. for *Pisaster giganteus.* Radial nerves were isolated and assayed from male or female animals in a ripe or spent condition. However, most of the experiments reported herein were performed using extracts obtained from the nerves of both male and female specimens taken during the spent condition.

The nerves were dissected as previously described (Chaet, 1964a) by severing all arms, removing the aboral surface, and making a light incision along the entire length of the ambulacral groove. The left and right halves were then separated, thus exposing the large pigmented radial nerve complex. The nerve was carefully removed with forceps, placed in chilled sea water and pooled with nerves of from 10 to 50 animals. The nerves were washed three times in chilled sea water and sometimes rinsed twice in ether. The nerves were then lyophilized, finely pulverized and stored dry until needed (a simpler, but less quantitative method of lysing the nerves has been described by Chaet and Musick, 1960).

Recipient starfish whose ripe gonads were to be used as assay material were carefully transported in chilled sea water to the laboratory and there maintained in refrigerated running sea water until required. The cold (13–14° C.) sea water not only kept animals from shedding their gametes during their natural shedding

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period, but decreased metabolic rates sufficiently so that little gonadal resorption occurred.

When gonadal fragments were to be used as assay tissue, the ovaries of *Asterias forbesi* or *Pisaster giganteus* were isolated by removing an arm of a ripe female and allowing the paired ovaries to float in a beaker of sea water. The ovaries were carefully dissected from the animal by severing the gonoduct. The gonads of *Patiria miniata* were exposed by performing an aboral mid-arm incision from the center portion of the disc to the tip of each of the 5 arms. The aboral surface was then folded back, and the exposed ovaries freed by cutting the gonoducts and suspending ligaments. In all three species, ovaries were either used intact or were cut into one-millimeter pieces to be employed in a semi-micro assay. In the latter case, cut pieces were washed in cold sea water until all free eggs had been washed away. In most experiments the fragments were used immediately. However, if kept refrigerated, the diced ovaries could be used 24–48 hours after removal from the animal, although re-washing was then necessary. Semi-micro assays were performed in 20 × 30 cm. clear, 96-hole, disposable spot plates containing 0.1 cc. of solution per depression. In all experiments, each individual assay was repeated a minimum of 8 times with ovarian fragments from two to four different animals. All assays contained proper controls, such as sea water, as well as a solution of standardized active shedding substance.

Semi-quantitative results were expressed by assigning a shedding value of from 0 to 5 to each piece of ovary at various times after immersing the tissue in the solutions to be assayed. The "shedding index" then calculated represented the quotient of actual shedding observed by maximum theoretical shedding (5 times the number of fragments).

When nerve extracts containing shedding substance were required, a 2 to 5 mg.% solution was prepared immediately before use by dissolving lyophilized nerve powder in fresh sea water. If storage was necessary, the solution was frozen since it was found that its biological activity was destroyed if salt water extracts were stored at room temperature (18 hours) or even merely chilled (5° C. for 7 days), whereas aliquots of the same extract were active for at least a year if kept frozen (−15° C.).

When fractionating samples of shedding substance, a 10-cc. saturated distilled water solution of lyophilized powder was centrifuged (15 minutes @ 10,000 RPM), and the supernatant collected. The precipitate resulting from the centrifugation was suspended in another 10 cc. of distilled water, centrifuged, and the supernatant pooled with the above. Two addtional washings were collected and pooled. The 40 cc. of supernatant were then lyophilized and redissolved in ½ cc. distilled water and placed on a Sephadex (G-25) column. Both short (17 cm. × 1.5 cm.) and long (60 cm. × 1.5 cm.) columns were used and run in a cold room at 7° C. The samples were eluted with water, collected in 4-cc. fractions, and immediately lyophilized. All fractions were simultaneously assayed on ovarian fragments after re-suspending in 2 cc. of fresh water.

RESULTS

Experiments in vivo

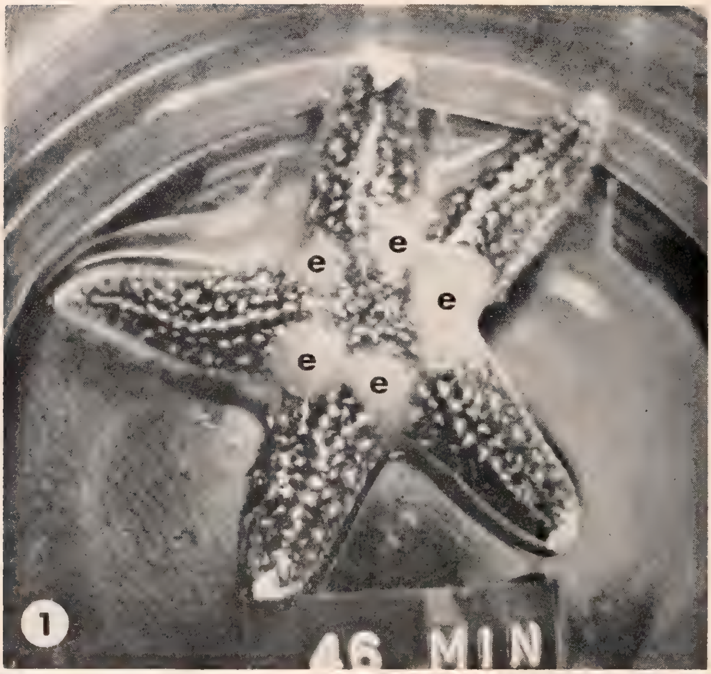
When extracts of *Asterias forbesi* radial nerves, prepared either by (1) heating an isolated nerve in 1 cc. of salt water for 1½ minutes at 76° C.; (2) lysing isolated nerves in distilled water, followed by replacement of salts (Chaet and Rose, 1961); or (3) adding powdered lyophilized nerves to sea water (5 mg.%), were injected (0.15 cc./gm.) into the coelomic cavity of ripe starfish, copious quantities of gametes were exuded through the gonopores (Fig. 1). Similar results were obtained by injecting radial nerve extracts from *Henricia sanguinolenta* into *Asterias forbesi* (Hartman and Chaet, 1962). When 2 mg.% sea water extracts were prepared from radial nerves of *Patiria miniata* and injected into the coelomic cavity of ripe *Patiria miniata,* eggs or sperm were released in large volumes (Fig. 2). Injections of control solutions very seldom elicited a shedding response.

It was noted that a lag period characteristically preceded the initial release of gametes. The precise duration of the interval was more predictable (30–35 minutes) in *Asterias forbesi,* than it was in *Patiria miniata* (25–40 minutes). The eggs released after an injection of shedding substance exhibited virtually complete (99%) germinal vesicle breakdown and were found to be highly fertile. The sperm released by injections of nerve extracts were motile and capable of fertilizing mature eggs.

Experiments in vitro

The presence of a lag period suggested the possibility that an intermediary substance was being formed which in turn stimulated the gonads, and that this over-all reaction required about 30 minutes. In an attempt to determine whether the gamete-shedding substance acted directly on the gonad or whether its action was indirect and involved other tissues of starfish, all 10 of the ovaries from a starfish were removed from the animal, leaving the gonopore, gonoduct, and gonad proper intact and uninjured. A small piece of the aboral surface surrounding the gonopore was left intact when isolating each ovary to insure that the gonoduct and gonopore were not injured. One-half of the gonads were then immersed in lyophilized nerve extracts (2 mg.% in sea water) and the other half in control solution (sea water). Isolated ovaries immersed in shedding substance released eggs which were seen to exude through the gonopore, but none was released from ovaries in sea water. Further, as was the case in all experiments involving intact animals, isolated gonads, or fragments of gonads (see below), shedding of gametes began only after a 30-minute interval. The above experiments were performed only on *Asterias forbesi* since it was surgically impractical to remove whole, intact, uninjured ovaries from *Patiria miniata.*

When ovaries were subjected to a threshold concentration of shedding substance, essentially all of the eggs were exuded through the gonopores within one to two hours. Figure 3 illustrates the magnitude of the reduction in gonad size after 1½ hours in shedding substance. All four gonads in Figure 3 were taken from the same starfish and were originally of equal size.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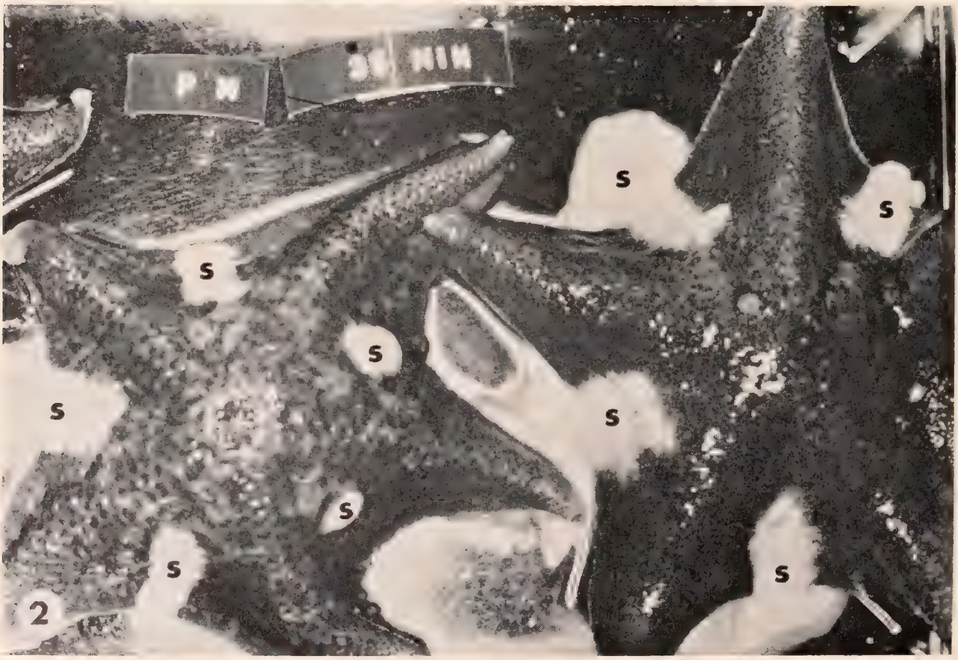
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To determine whether eggs must pass through the gonoduct to insure their complete maturation, ovaries from the same animal were carefully isolated, keeping 5 ovaries intact and puncturing the remaining 5 at various levels from the proximal to the distal ends of the gonads. Both intact and punctured ovaries were then placed in shedding substance, and the results of a typical experiment are shown in Table I. In both cases shedding began 30 minutes after immersion in the neural extract containing shedding substance. Eggs were exuded (shed) from the intact ovaries only through the gonopore, whereas in the punctured ovaries, eggs were exuded mainly through the punctured areas, very few leaving through the gonopore. The disappearance of germinal vesicles (final maturation) was determined microscopically and, as can be seen in Table I, the eggs leaving the ovary through the punctured sites exhibited as high a percentage of germinal vesicle breakdown as did those eggs passing through the gonoducts and gonopores. It should also be noted that not only was shedding completed first in the punctured ovaries, but larger volumes of eggs were released from the punctured gonads than from unpierced specimens when both were immersed in the same concentration of shedding substance.

TABLE I

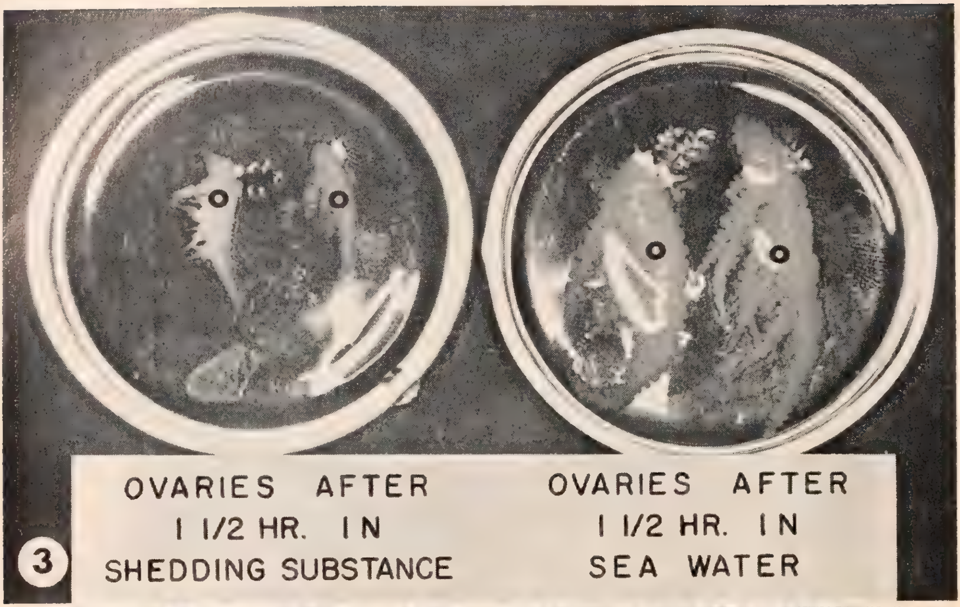
A typical experiment in which 10 ovaries of Asterias forbesi were immersed for 1½ hours in 10 mg.% solution of nerve extract. Five "intact ovaries" had only their gonoducts severed, whereas the 5 "punctured ovaries" were also punctured in several areas from the proximal to distal end of the ovary. Each figure represents an average of 5 gonads.

Sister gonads immersed in shedding substance			
Intact ovaries		Punctured ovaries	
Quantity of eggs shed (max. value = 5)	Germinal vesicle breakdown	Quantity of eggs shed (max. value = 5)	Germinal vesicle breakdown
2.2	98%	4.8	100%

Since it was found that passage of eggs through the gonoduct was not necessary for complete maturation, and since a micro-assay for determining the presence and characteristics of shedding substance was desirable, the next series of experiments involved cutting isolated ovaries into small fragments (Chaet, Andrews and Smith, 1964). Ovarian fragments, thoroughly washed in sea water, were immersed in 0.1 cc. of experimental or control solutions. Shedding began only in those solutions containing shedding substance and as was observed in all previous experiments, only after a 30-minute interval. Figure 4 illustrates a portion of the picture seen in a typical ovarian fragment experiment. Note the accumulation of eggs around the ovarian fragments immersed in gamete-shedding substance (s.s.) and the lack of eggs around fragments immersed in control solutions (s.w.).

FIGURE 1. Eggs (e) exuding from gonopores of a female *Asterias forbesi* 46 minutes after receiving an intracoelomic injection (5 mg.%—0.15 cc./gm.) of radial nerve extract containing shedding substance.

FIGURE 2. Sperm (s) exuding from gonopores of *Patiria miniata* 35 minutes after injection (2 mg.%) of nerve extract containing shedding substance.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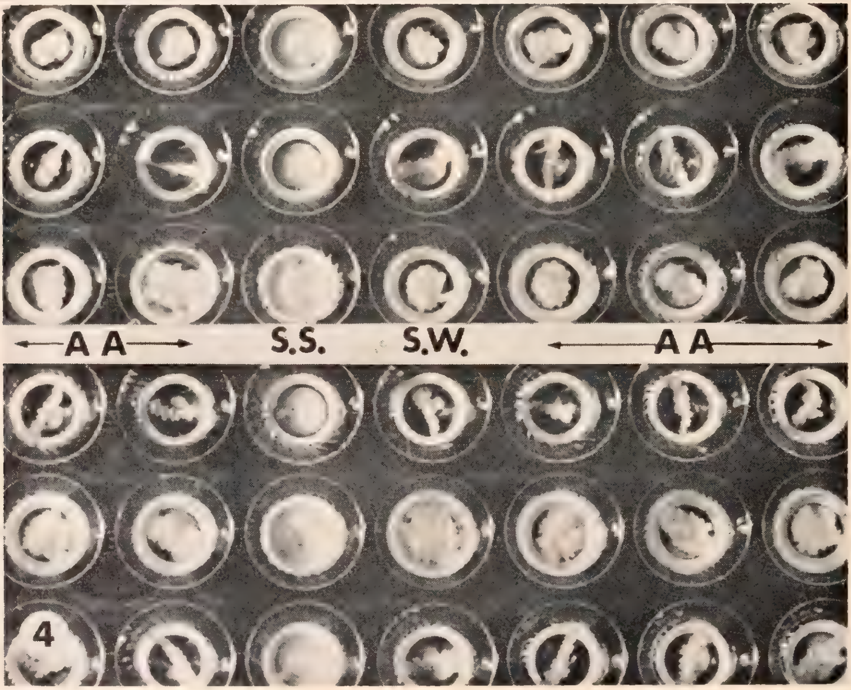

FIGURE 3. Comparison of four sister-ovaries (o) after two were immersed in nerve extract containing shedding substance and had shed their eggs. Ovaries transferred to fresh sea water after 1½ hours in original solutions.

Concentration of shedding substance

By serial dilution assay of lyophilized nerves from both sexes of *Asterias forbesi,* it was found not only that shedding substance was present in nerves of both sexes, but that nerves from male and female contained identical concentrations of shedding substance. Experiments using intact starfish showed that shedding substance taken from nerves of either sex stimulated shedding in both sexes.

Radial nerves taken from *Asterias forbesi* before natural shedding had occurred (May and June) were assayed and the concentration of shedding substance was then compared with that found in nerves taken from starfish after they had naturally shed (July and August). Again serial dilution assays demonstrated that the level of shedding substance was the same in both instances. To further examine this point, monthly collections of starfish (*Asterias forbesi*) were made for a period of two years. Chilled animals were flown (8–20 hours) to the laboratory and the nerves removed, lyophilized and stored until all of the samples could be assayed simultaneously. The results of these experiments illustrated that the level of shedding substance remained constant throughout the year, and that no peak accumulation of gamete shedding substance was found even in those radial nerves that were taken from animals just prior to natural shedding. By chance, a few specimens arrived while they were shedding their gametes. Their assayed nerves revealed the same level of shedding substance as was found in the monthly samples (pre-shed and spent conditions).

Specificity of shedding substance

The shedding substance was found in the radial nerves of 10 echinoderms from both the east and west coasts of the United States. Originally demonstrated in *Asterias forbesi*, the substance was later found in the radial nerves of *Henricia sanguinolenta* (Hartman and Chaet, 1962). Radial nerve extracts from *Patiria miniata* and *Pisaster giganteus*, both found on the west coast, were found to contain shedding substance (Chaet, 1964b); this also applies to *Pisaster ochraceus, Pisaster brevispinus, Astropecten armatus, Pycnopodia helianthoides, Heliaster kubinijii* and *Orthasterias koehleri*). The various chemical characteristics that have been measured suggest similarities between the shedding substances in all 8 species. Although all cross-specificity experiments have not been completed to date, it was found that the shedding substance of *Asterias forbesi* was active on the ovaries of *Pisaster giganteus, Heliaster kubinijii, Pisaster ochraceus,* but not on *Patiria miniata* or *Astropecten armatus*. *Henricia sanguinolenta* was assayed on *Asterias forbesi* (Hartman and Chaet, 1962) and found to release eggs. The shedding substance from *Patiria miniata* was active on ovaries of *Pisaster giganteus, Heliaster kubinijii, Pisaster ochraceus* and *Astropecten armatus*. The shedding substance from *Pisaster giganteus* and *P. ochraceus* induced the release of eggs from the ovaries of *Heliaster kubinijii, Patiria miniata* and *Astropecten armatus*. *Pycnopodia helianthoides* shedding substance was found to be active on *Heliaster*

FIGURE 4. A portion of the ovarian fragment assay (*Asterias forbesi*) performed in 96-hole spot plate. Compare columns "shedding substance" (SS) and "sea water" (SW). The remaining columns contain various amino acids (AA), none of which induced shedding.

TABLE II

Relationships of biological specificity between various starfishes. In vitro ovarian fragment assay was used in which 4 mg.% and 10 mg.% solutions were tested. (When shedding did not occur, both higher and lower concentrations were also assayed.)

	Radial nerves from:									
	Asterias forbesi	*Astropectin armatus*	*Heliaster kubinijii*	*Henricia sanguinolenta*	*Orthasterias koehleri*	*Patiria miniata*	*Pisaster brevispinus*	*Pisaster giganteus*	*Pisaster ochraceus*	*Pycnopodia helianthoides*
Assay tissue from:										
Asterias forbesi	Shed			Shed						
Astropectin armatus	None					Shed		Shed		None
Heliaster kubinijii	Shed	Shed	Shed		Shed	Shed	Shed	Shed	Shed	Shed
Patiria miniata	None	None	None		Shed	Shed	Shed	Shed	Shed	None
Pisaster giganteus	Shed							Shed	Shed	
Pisaster ochraceus	Shed					Shed		Shed	Shed	Shed

kubinijii, Pisaster ochraceus, but not on *Patiria miniata* or *Astropecten armatus.* Table II summarizes these findings.

Physiological mechanism

Several types of experiments were designed to help uncover the specific mechanisms believed to be useful in explaining the physiological action of shedding substance on ovaries (for technical reasons, these have not been extensively studied on the testes). The final result of an ovary exposed to shedding substance for one to two hours (see Fig. 3) suggested that (1) the ovary contracted and forced out its gametes from within the gonad, or (2) the eggs were released from the ovary by some other mechanism and as a result of the elasticity of the ovarian wall, the ovary contracted after the eggs had left the gonad. To investigate these alternative possibilities, the over-all length of isolated uninjured ovaries, while suspended in a sea water solution containing shedding substance, was constantly measured through the aid of a myograph-transducer, amplifier, and chart recorder (Chaet, Andrews and Smith, 1964). Figure 5 is a reproduction of a typical result obtained in such an experiment. It should be noted (Fig. 5) that the ovary began to contract at least 25 minutes after the addition of shedding substance—some 2–3 minutes (in some experiments, 5–10 minutes) before eggs were seen to be exuded through the gonopore. These findings suggested that one physiological action of the radial nerve extract containing shedding substance was due to a "contraction factor," which stimulated ovarian contraction, resulting in the active expulsion of eggs. It might be suspected that if contraction played a role in the shedding process, ionic calcium might be implicated in this over-all shedding reaction. It was found (Mecklenburg and Chaet, 1964) that ovarian fragments placed in calcium-free sea water containing shedding substance failed to release their eggs even if kept in such solutions for several hours. However, when these ovarian fragments were removed, washed, and placed in normal sea water (containing calcium but no shedding substance), the tissues immediately began to shed.

A second type of experiment was designed to explain the fact that 98–100% of the eggs released as a result of shedding substance activity exhibited germinal vesicle breakdown and thus had completed maturation. To study this phenomenon, sister-ovaries were immersed in sea water or shedding substance solutions for one hour. After that time, the gonads were placed in sea water, minced with fine scissors, filtered through cheesecloth, and the eggs in the filtrate were washed in fresh sea water. Microscopic examination illustrated that the "sea water-ovary" contained small immature eggs, large immature eggs and large mature eggs (no eggs were exuded from these ovaries into the sea water). In the remains of the "shedding substance-ovary," only small immature eggs (as well as some malformed large immature eggs) were present, and typical large immature eggs were for the most part absent. As noted above, large mature eggs had been shed into the surrounding medium. The absence of large immature eggs suggested that their conversion to mature eggs was stimulated by the presence of a "maturation factor" in nerve extracts.

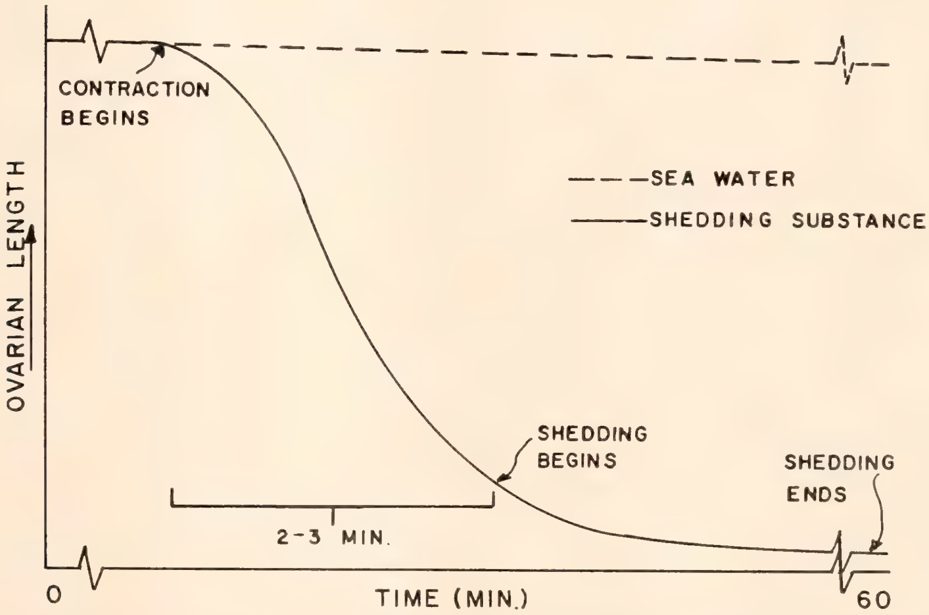

FIGURE 5. Plot of experiment designed to determine if eggs were released from isolated-intact ovaries before or after ovarian contraction. The length of *Asterias forbesi* ovaries was constantly recorded while immersed in 5 mg.% solution of radial nerve extract in sea water, or sea water alone.

To date, all attempts to separate the "contraction factor" from the "maturation factor" have been unsuccessful.

Neurosecretion

Various tissues of *Asterias forbesi* were assayed for the presence of gamete-shedding substance. Hot (76° C.) salt water extracts were prepared from the digestive tract, tube foot, oral and aboral surface, coelomic fluid, gonads, and hepatic caeca. The extracts were assayed on starfish ovaries and found to possess no gamete-shedding activity. Gamete-shedding activity was found only in hot salt water extracts of radial nerve. Since electron micrographs of the radial nerve suggested that the structure from which shedding substance had been extracted was indeed neural tissue, an attempt was made to search for neurosecretory granules in the radial nerve complex of *Asterias forbesi.* Typical neurosecretory stains, such as performic acid alcian blue, paraldehyde-fuchsin, chromalum-hematoxylin-phlozine and azan (Imlay and Chaet, 1965), demonstrated neurosecretory-like granules, 1 μ and 2 μ in diameter, present in the ventral layer of the radial nerve. The neurosecretory substance was positively correlated with the presence of active gamete-shedding substance, thus suggesting the possibility that shedding substance be placed in the category of a neurosecretion. Other experiments (Uter, 1966) in which radial nerves were stripped into layers showed that active gamete-shedding substance was located only in the most aboral region of the nerve—an area known to be rich in neurosecretory-like granules.

Chemical nature of shedding substance

The shedding substance from *Asterias forbesi* and from *Patiria miniata* was subjected to limited chemical analysis. It was found to be adsorbed on Dowex-50, which first suggested the possibility of a basic polypeptide. Further experiments in which shedding substance was incubated in 0.1% trypsin (twice crystallized)

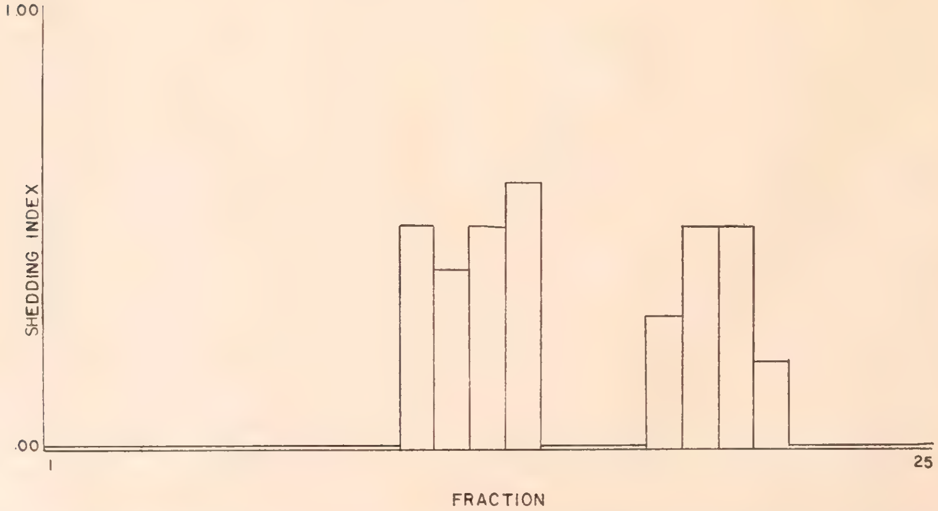

Figure 6. Biological activity of *Patiria miniata* shedding substance fractionated with Sephadex G-25 (58 cm. long.) columns. Note the two distinct peaks, a and b.

at pH 7 for 4 hours at 40° C. demonstrated that biological activity of shedding substance was lost after tryptic digestion, suggesting its polypeptide nature (Chaet, 1964c). It was also found that the addition of 0.2% ninhydrin to gamete-shedding substance resulted in a loss of biological activity.

Analysis of distilled water extracts from *Asterias forbesi* nerves by thin layer chromatography suggested the possible presence of 17 different amino acids and/or 19 dipeptides. Thus, various concentrations of known amino acids and dipeptides (chosen according to their R_f values) were assayed using the ovarian fragment spot-plate system. Stock amino acids were assayed for shedding activity, both as individual amino acids and in various combinations. However, none of the amino acids (individually or in combination) resulted in shedding activity. Figure 4 illustrates a typical example of one such assay involving 5 amino acids. An attempt was also made to mimic shedding activity with follicle-stimulating hormone, luteinizing hormone, 4-aminobutyric acid, and dl-2-aminobutyric acid by injection into intact starfish, but none induced the release of gametes.

To further purify the shedding substance, distilled water extracts of lyophilized *Patiria miniata* nerves were fractionated on 58-cm.-long Sephadex (G-25) columns and eluted with distilled water. Each 2-ml. fraction was then lyopholized, dissolved in sea water, and assayed on ovarian fragments. The result of a typical experiment was illustrated in Figure 6, which clearly demonstrated that two separate peaks were obtained, both possessing shedding activity. The above separation was observed, although less pronounced, when using 17-cm.-long columns.

Shedding substance purified on Sephadex was subjected to ninhydrin titer (Moore and Stein, 1954) before and after acid hydrolysis in sealed vials heated to 110° C. for 12 hours. Since hydrolysis of purified shedding substance only resulted in a four-fold increase in absorption (570 mμ) after the addition of ninhydrin and hydrindantin in methyl cellosolve and sodium acetate at pH 5.5, it appeared that the shedding substance was a polypeptide consisting of 10–15 amino acids.

Inhibitory substance

Experiments with *Asterias forbesi* and with *Patiria miniata,* involving various salt water concentrations (0.1 to 100 mg.%) of lyophilized nerves taken from pre-shed (ripe) starfish, showed that, whereas low concentrations of extract (2–5 mg.%) caused shedding, higher concentrations (10–100 mg.%) failed to cause shedding. If, on the other hand, the nerves were taken from post-shed (unripe) animals, high (10–100 mg.%) as well as low concentrations induced shedding. The results of a typical experiment may be seen in Table III. Since these findings suggested the presence of an inhibitory substance in the nerves taken from ripe animals, which was present at a threshold level only at the higher concentrations, nerve extracts from ripe starfish (which did not induce shedding in high concentrations) were fractionated (4-mg. aliquots) by passing over either long or short Sephadex G-25 columns. Once lyophilized, all fractions were redissolved in 2 ml. sea water, mixed with an equal volume of a fraction previously assayed and known to possess shedding activity, and each fraction was then subjected to ovarian fragment assay. The result of a typical assay experiment (using a short column) is graphically shown in Figure 7, in which one fraction was found (#5)

TABLE III

Assay of nerves obtained from pre- and post-shed Patiria miniata.
Figures expressed as shedding index.

	Lyophilized Nerve Extract (in sea water)				
	0 mg.%	5 mg.%	10 mg.%	25 mg.%	100 mg.%
Pre-shed (ripe)	0.00	0.30	0.05	0.00	—
Post-shed (unripe)	0.00	0.53	0.53	0.55	0.25

that inhibited shedding of ovarian fragments even though all fragments were under the influence of active purified shedding substance (#3). Since this fraction inhibited shedding activity, it was called "shedhibin." Similar assays were performed using extremely large ovaries from maximally ripe starfish (*Patiria miniata*). Such ovarian fragments, provided that they were taken from very ripe females, shed spontaneously (even in the absence of added shedding substance) within 15 to 30 minutes after immersion in sea water. However, when these fragments were immersed in each of the above fractions taken from Sephadex columns, all fractions allowed shedding to occur except that fraction (#5) previously found to contain shedhibin. No shedhibin could be detected in those radial nerves taken from post-shed, unripe females whose crude nerve extract induced shedding at higher concentrations.

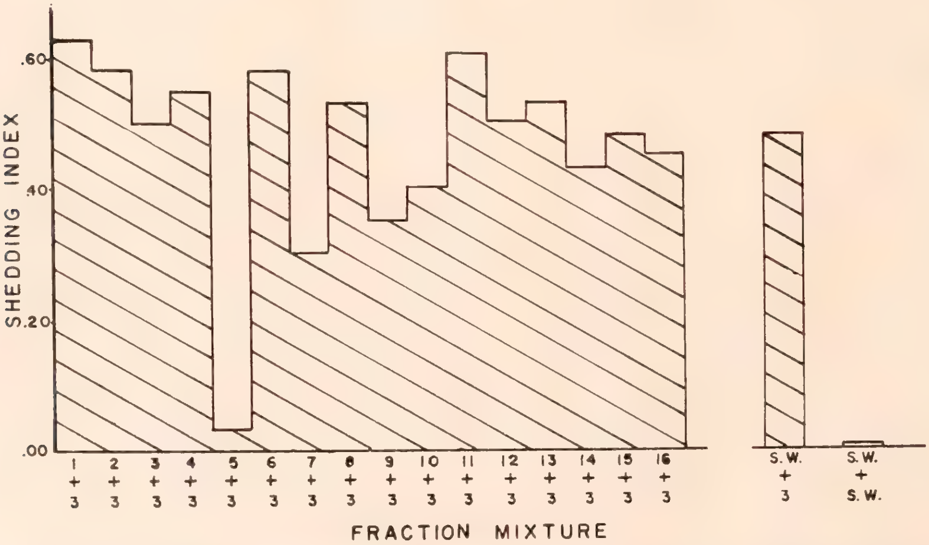

FIGURE 7. Graphic representation of *Patiria miniata* ovarian fragment assay, illustrating presence of shedhibin in fraction #5 of fractionated *Patiria miniata* radial nerves. Five-ml. fractions eluted from short Sephadex G-25 column were assayed after mixing with an equal volume of fraction #3, known to contain shedding substance.

Discussion

The gamete-shedding substance(s) found in the radial nerve of sea-stars was shown to be responsible for inducing the release of mature eggs or sperm when injected into ripe starfish of the same species. Figures 1 and 2 illustrate the release of gametes from two different species, *Asterias forbesi* from the Atlantic coast and *Patiria miniata* from the Pacific coast. Actually a total of 14 different species of sea-stars have now been found to contain gamete-shedding substance. In addition to those listed in Table II shedding activity has been found in *Asterias glacialis* (Unger, 1962), *Asterias amurensis, Astropecten scoparius and Coscinasterias acutispine* (Kanatani and Noumura, 1962). The shedding substance was not species-specific as Hartman and Chaet (1962) first demonstrated when nerve extracts from *Henricia sanguinolenta* were injected into *Asterias forbesi,* resulting in the release of mature gametes. Later, extracts of *Asterina pectinifera* were found to induce spawning in *Astropecten scoparius* or *Coscinasterias acutispina* (Kanatani and Noumura, 1962). On the other hand, a certain degree of species specificity does exist, since, for example, shedding substance from *Asterias forbesi* or *Pycnopodia helianthoides* did not induce shedding when assayed on isolated ovarian fragments of *Patiria miniata* or *Astropecten scoparius,* although extracts from *Pisaster giganteus* and *P. ochraceus* (as well as *Patiria miniata*) proved highly active in *Patiria* ovaries.

It should be noted that in experiments both *in vivo* and *in vitro* it was consistently found that a 30-minute interval was evident—that is, eggs were not shed until 30 minutes after injection of nerve extracts into the coelomic cavity of an intact starfish (Chaet and Musick, 1960), or immersion of an isolated ovary (Chaet and Smith, 1962), or fragment (Chaet, Andrews and Smith, 1964) into a solution of shedding substance. Since experiments *in vitro* involving isolated ovarian fragments demonstrated that the lag period persisted, it was concluded that the shedding substance acted directly on the ovarian tissue itself and might be considered a "first order neuro-endocrine" mechanism as defined by Rothballer, (1957).

Since the onset of ovarian contraction began some 2–3 minutes before shedding actually occurred (Fig. 5), it was theorized that the shedding substance contained a "contraction factor." Further evidence supporting this theory of a contraction factor was seen in those experiments which implicated the role of calcium on the physiological activity of the shedding substance (Mecklenburg and Chaet, 1964). If the shedding substance did indeed act on ovarian muscle, causing an over-all contraction and forcing-out of gametes, it would be expected, according to theories involving muscle contraction (Weber, 1958), that ovarian contraction and, therefore, shedding would not occur in the absence of calcium even though ample quantities of shedding substance were present. It might also be expected that when calcium was again added, shedding would begin. This was found to be the case when using ovaries and shedding substance from both *Asterias forbesi* and *Patiria miniata* (Mecklenburg and Chaet, 1964). The inability of shedding substance to function in the absence of calcium was confirmed for *Asterias amurensis* (Kanatani, 1964).

The presence of a "maturation factor" (Chaet, 1964c; Chaet, Andrews and

Smith, 1964) was suspected by comparing gametes remaining in sister ovaries (*Asterias forbesi*) after immersion in gamete-shedding substance or control solutions. These findings have been confirmed on a second species of sea-star, *Asterias amurensis* (Kanatani, 1964). In addition to contraction and maturation factors previously reported by Chaet and co-workers, Kanatani (1964) has suggested that still another action of shedding substance was to free eggs from cohering to each other and/or from adhering to the ovarian wall.

It was first noted by Chaet and Smith (1962) that the concentration of shedding substance in nerves taken from ripe starfish contained the same concentration of shedding substance as did nerves of an unripe (spent) animal. Further, monthly nerve samples collected over a two-year period, when simultaneously serially assayed, again indicated a constant level of shedding substance in the radial nerve of *Asterias forbesi.* Noumura and Kanatani (1962) reported that a seasonal variation did occur; however, they did not measure the concentration or physiological activity of shedding substance (personal communication). They reached their conclusion after noting a change in transparency of nerves and also pointed out (p. 400) that "histological studies also revealed seasonal changes in the radial nerve, as will be reported elsewhere." Imlay and Chaet (1965) independently noted seasonal variations after studying histological sections of radial nerves stained with "neurosecretory stains," but they could not conclude that these histological variations were in any way correlated with the presence or a change in level of shedding substance. It is possible that the seasonal variation noted may reflect the level of shedhibin, but we have yet to study this point.

Since the level of shedding substance remained constant throughout the year, it was indeed difficult to surmise the role of gamete-shedding substance in natural spawning. However, it was noted when working with *Asterias forbesi* and later with *Patiria miniata* (Table III) that shedding failed to occur when higher concentrations of nerve extracts were used, provided that they were taken from nerves of ripe animals. These findings then stimulated a search for an inhibitor, and at the present time, the inhibitor "shedhibin" was found only in nerves taken from ripe starfish and was absent in nerves taken from spent starfish. Studies are underway to determine the precise level of shedhibin in nerves of *Patiria miniata* by collecting bimonthly specimens. Since high concentrations (10 mg.%) of nerve extracts prepared from nerves of post-shed (unripe) animals continued to induce shedding, and since shedhibin was isolated from nerves of ripe starfish, but could not be found in unripe animals, the obvious suggestion arose that natural control of shedding was regulated both by the level of shedding substance and the presence (or absence) of shedhibin. It appears that a seasonal disappearance of shedhibin may be responsible for the release of gametes in the presence of shedding substance.

The physiological significance of two distinctly different fractions of shedding substance of different molecular weight (Fig. 6) has not been explained and may merely represent an artifact, but it is of interest that both substances remained biologically active. Whether the shedding substance complexed with other molecules of different size or whether the shedding substance has been split into a smaller molecule which maintained its physiological activity when degraded has yet to be determined. Kanatani and Noumura (1962) independently reported that they concentrated shedding substance on Sephadex columns, but found only one peak rather than the two reported by Chaet (1964b). This was understandable since they used columns only 35 cm. in height, which, according to our own experiments, would not adequately separate the two peaks. They also reported an ability to concentrate shedding substance, a phenomenon which may also be explained merely on the basis that they unknowingly had removed shedhibin which inhibited their shedding substance activity.

It should be noted that granules were observed (Imlay and Chaet, 1965) which stained positively with four different neurosecretory stains. Some, but not all, of the granules were present during pre- and post-shedding stages. Granules staining with the neurosecretory stains were present in the most ventral layer of radial nerves of *Asterias forbesi* (Imlay and Chaet, 1965), which was the only area of the radial nerve complex in which physiologically active shedding substance was found (Uter, 1966). Using the most critical criteria of neurosecretion (Bern, 1963), it is difficult to place conclusively the shedding substance in the realm of a neurosecretory substance, and the above findings merely suggest that the granules are neurosecretory in nature. The studies of Unger (1962) as well as studies of Philpott and Chaet (unpublished), involving the electron microscopy of radial nerves of two asteroids, as well as those light microscopy studies of Noumura & Kanatani (1962), all of which illustrated the presence of "secretory granules," continue to suggest that the shedding substance represents neurosecretory activity.

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SUMMARY

1. A neurochemical substance from the radial nerve of *Asterias forbesi* and *Patiria miniata* has been studied *in vivo* and *in vitro* and found to initiate both gamete release and maturation. The significance of a 30-minute lag period before gamete release, which was evident in all experiments, was discussed.

2. Gamete release and/or maturation did not depend upon passage through the gonoduct or gonopore.

3. The level of shedding substance in radial nerves of starfish was found to be constant throughout the year, and was found in a total of 10 different species. Cross-experiments indicated that a limited degree of species specificity existed.

4. The possibility that the shedding substance is a neurosecretory product was discussed.

5. The shedding substance was found to be a polypeptide, consisting of 10–15 amino acids, which was purified for future amino acid analysis.

6. An inhibitor to the shedding substance, shedhibin, was also found in the radial nerve. Its presence varied with the reproductive state of the animal.

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THE INTERRELATIONS AMONG THREE HORMONAL-CONTROLLED CHARACTERS IN THE ADULT FEMALE SHORE CRAB, CARCINUS MAENAS (L.)

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The three decapod characters, molting, ovarian developing stages and shell color changes, have all been shown to be under hormonal control (Brown and Jones, 1947; Carlisle, 1953a, 1953b; Démeusy, 1962; Echalier, 1955, 1956; Lenel and Veillet, 1951; and Passano, 1951b, etc.).

While much experimental work has been done by the above authors on this field, so far no attempt has yet been made to investigate the interrelation among these characters under natural conditions. This study was therefore carried out in the Department of Zoology, University of Glasgow by examining specimens of female *Carcinus maenas* (L.) collected from Millport, Isle of Cumbrae between November, 1961, and March, 1964.

MATERIALS AND METHODS

The crabs were taken in baited creels off the Millport Marine Laboratory near Old Pier at a depth of 3 to 4 meters. At least one lot of crabs was taken nearly every month so that the study included examples in all seasons. Crabs with internal or external *Sacculina* were discarded, since the effect of parasitic castration would retard normal ovarian growth (Charniaux-Cotton, 1960, p. 419), inhibit molting (Passano, 1960, p. 483) as well as absorb carotenoid pigments (Lenel, 1954).

All the crabs were adults with carapace width greater than 40 mm. The number of female crabs included in this study was 519, but the conclusions reached are found to be statistically significant in nearly all cases.

Classification of the stages of the ovary was made according to the following scheme:

White. The ovary was redeveloping after the spent stage. In section, active proliferation was visible. Resorption of yolk and degeneration of old oocytes from the last spawning were completed. In many cases the crab could be recognized as having been recently fertilized after the last molt, the spermathecae appearing swollen.

Yellow. A stage subsequent to white. The yellow colors are due to yolk deposition. The ovary had enlarged considerably but was far from its full size.

Orange. At this stage yolk deposition had gone on still further so that the ovary acquired its orange color. The ovary had enlarged and it was during this stage that it reached full size and was ready for egg-laying.

Spent. This included crabs that had spawned recently, but mostly had not yet undergone a molt since. All berried crabs were in this category. The ovary was here reduced in size, with still a few mature oocytes.

Classification of the molting stages followed that of Passano (1960, p. 477) which is a modified scheme of Drach's (1939). Since this classification is well known, a reiteration here is not necessary. Because of the fact that the crabs were collected in baited creels the non-feeding stages D_3, D_4 (later premolts), E (molting) as well as A_1, A_2 and B_1 (early postmolt) were missing from the sample throughout the study. Therefore, the above detailed classification had to be modified in a convenient way for this particular work. Accordingly, the crabs were divided into (a) postmolt, which included stages from B_2 to C_3, (b) intermolt, including only C_4, and (c) premolt, which consisted of D_0 to D_2 crabs. The above-mentioned missing stages around molting should be very short (Passano, 1960) in the molting cycle so that conclusion in this work would not be affected.

The color stages were classified on the basis of the color on the ventral surface of the animal excluding the carapace, but including the abdomen in its natural position. These were green, yellow and orange-red; occasionally blue and brown colors were found present with the above. The latter two colors, especially blue, when present usually occupied much smaller areas than the first three, which might be present on the whole of the ventral side. In cases where two colors were found the crab was recorded as belonging half to one and half to another color; similarly, when three colors occurred in the same crab it was recorded as one-third belonging to each of the three colors, irrespective of the proportion in areas each color occupied. The numbers of crabs recorded in Tables III and V were taken to the nearest halves. As the blue and brown colors occurred but rarely (Table III), they are not represented in Figure 2.

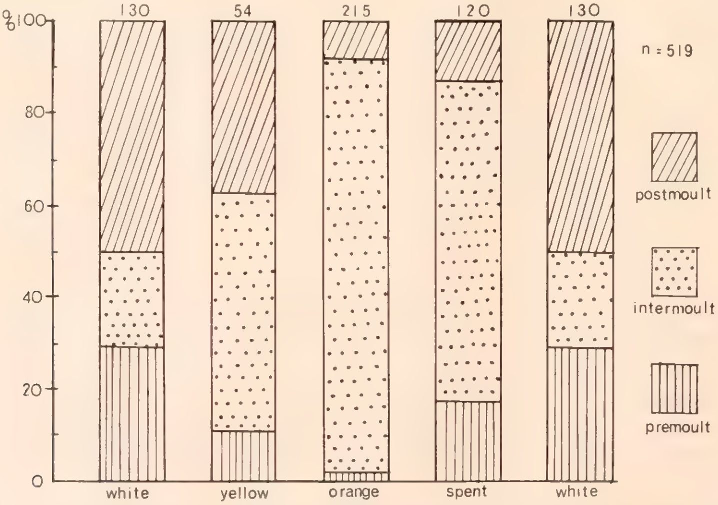

FIGURE 1. Relation between various ovarian and molting stages. Abscissa: different stages of the ovaries (see text). Postmolt = stages B_2–C_3; intermolt = stage C_4; premolt = stages D_0–D_2.

TABLE I

Relation between various ovarian and molting stages. Postmolt = stages B_2-C_3; intermolt = stage C_4; Premolt = stages D_0-D_2. Percent in bracket.

	Ovary				Total
	White	Yellow	Orange	Spent	
Postmolt	65 (50.0)	20 (37.0)	17 (7.9)	15 (12.5)	117
Intermolt	27 (20.7)	28 (51.8)	194 (90.2)	84 (70.0)	333
Premolt	38 (29.2)	6 (11.1)	4 (1.9)	21 (17.5)	69
Total	130 (100)	54 (100)	215 (100)	120 (100)	519

These colors were apparently due to pigments in the cuticular layers in the integument of the animals. The biochemical nature of the changes of coloration in the *Carcinus* shell has been worked out in some detail by Lenel (1953, 1954, 1955) and Lenel and Veillet (1951).

RELATION BETWEEN OVARIAN AND MOLTING CYCLES

The proportions of the three molting stages present in each of the four ovarian stages are represented in Figure 1 and Table I. The exclusion of certain stages around molting does not affect, however, the following conclusions, which are also found to be sound statistically by the Chi-square method, using Yate's correction (Table II). (1) In the category of white ovaries, pre- and postmolt crabs predominated, whereas intermolt crabs were rare. (2) As the developmental stages of the ovaries changed through yellow to orange, the proportion of pre- and postmolt crabs decreased, while that of intermolt increased, until at orange the first two were at minimum and the last one was at maximum. At the spent stage the tendency was reversed but the intermolt crabs were still predominant. However, a significant rise in premolt individuals occurred, until at the white stage this, together with the postmolt, was at maximum.

TABLE II

Test of significance by the "Chi-square Method," with Yate's correction, on the relation between ovarian and molting stages in adult female Carcinus. W = white; Y = yellow; O = orange; Sp = spent. P = probability in per cent. Postmolt includes B_2-C_3; intermolt C_4; premolt D_0-D_2 crabs. Stages D_3-B_1 around molting were excluded in the study (see text).

Change in molting stages	Change in stages of ovaries			
	W to Y	Y to O	O to Sp	Sp to W
Postmolt to intermolt	$P < 1$	$P < 0.1$	$P \div 5$	$P < 0.1$
Intermolt to premolt	$P < 0.1$	$P < 0.1$	$P < 0.1$	P about 0.1
Premolt to postmolt		(not tested)		

OVARIAN STAGE AND SHELL COLOR

The more commonly occurring shell colors on the ventral side of the animal are given with the corresponding ovarian stage in Table III and their percentages in Figure 2A. Shell colors are green, yellow and orange-red. In the immature white gonad stage, green was found to be the most frequent shell color. As the ovary became yellow, the frequency of green shell color decreased whereas the yellow and orange-red increased. At the orange ovarian stage both yellow and green shell frequency decreased while orange-red increased. This latter tendency continued to the spent stage.

TABLE III

Relation between various ovarian stages and shell color. Per cent in brackets.

Shell color	Ovary				Total
	White	Yellow	Orange	Spent	
Green	73 (57.3)	18½ (34)	35½ (16.7)	6 (5)	133
Yellow	20 (15.7)	14½ (26.6)	33½ (15.8)	7½ (6.3)	75.5
Orange-red	29½ (23.1)	19½ (35.7)	138 (65)	98 (82)	285
Brown	4 (3.1)	0 (0)	2 (0.9)	1½ (1.3)	7.5
Blue	1 (0.8)	2 (3.7)	3½ (1.65)	6½ (5.4)	13
Total	127½ (100)	54½ (100)	212½ (100)	119½ (100)	514

Chi-square tests with Yate's correction indicated significance in the above changes (Table IV).

TABLE IV

Test of significance by the "Chi-square Method" with Yate's correction on the relation between ovarian stages and shell color in adult female Carcinus. W = white; Y = yellow; O = orange; Sp = spent.

Change in color on shells	Change in stage of ovaries			
	W → Y	Y → O	O → Sp	Sp → W
Green to orange-red	$P = 5$	$P < 0.1$	$P < 1$	—
Orange-red to green	—	—	—	$P < 1$
Green to yellow	$P < 5$	—	—	—
Yellow to orange-red	—	$P < 0.1$	$P < 1$	—
Orange-red to yellow	—	—	—	$P < 0.1$

We should therefore accept that the change in each of the above-mentioned cases is significant, and arrive at the conclusion that the green shell color, present most abundantly in the white ovarian stage, turns to yellow and orange-red as the crab's ovary becomes fully mature and spent.

Here the yellow appeared to be transitional shell color at the transformation between green and orange-red.

MOLTING AND SHELL COLOR

The numbers of green, orange-red and yellow shell colors included in the three molting stages are included in Table V and their percentages plotted in Figure 2B. Here at postmolt more green than orange-red or yellow was present. At intermolt, orange-red increased sharply while the other two decreased. At premolt, orange-red and yellow decreased while green increased.

TABLE V

Relation between molting stages and shell color. Postmolt = stages B_2-C_3; intermolt = stage C_4; Premolt = stages D_0-D_2. Per cent in brackets.

	Postmolt	Intermolt	Premolt	Total
Green	54 (42.4)	55 (17)	24 (37.8)	133
Yellow	36 (28.2)	35 (10.8)	4½ (7.1)	75½
Orange-red	27 (21.1)	226 (70)	32 (50.4)	285
Brown	3 (2.3)	3½ (1.8)	1 (1.6)	7½
Blue	7½ (6)	3½ (1.8)	2 (3.2)	13
Total	127½ (100)	323 (100)	63½ (100)	514

Similar statistical tests are summed up in Table VI.

The contrasting occurrence of green and orange-red is therefore established in the molting as well as maturing cycles. From Figure 2B, it can be seen that most intermolt crabs have orange-red shells. Further (Fig. 1), more intermolt crabs were found with mature (orange) rather than spent ovaries. Yet (Fig. 2A) a smaller proportion of crabs with orange-red were found with mature (orange) ovaries than spent. This was due to the fact that crabs with orange ovaries included a significantly higher proportion of green and yellow (especially green) shells (Fig. 2A and Table III).

TABLE VI

Test of significance by the "Chi-square Method", with Yate's correction, on the relation between molting stages and shell colors in adult female Carcinus crabs. Postmolt includes B_2-C_3; intermolt C_4; premolt D_0-D_2 crabs.

Change in molting stages	Change in color of shells	
	Green to orange-red	Orange-red to yellow
Postmolt to intermolt	$P < 0.1$	$P < 0.1$
Intermolt to premolt	$P < 1$	P about 70
Premolt to postmolt	P about 1	P about 0.1

It was noted that crabs smaller than about 35 mm. (not included in this study) carapace width had much less orange-red on the shells than larger ones. They appeared green in color at most stages, though at premolt, additional blue tinges on certain parts of the shell could be distinguished. When newly molted, in the

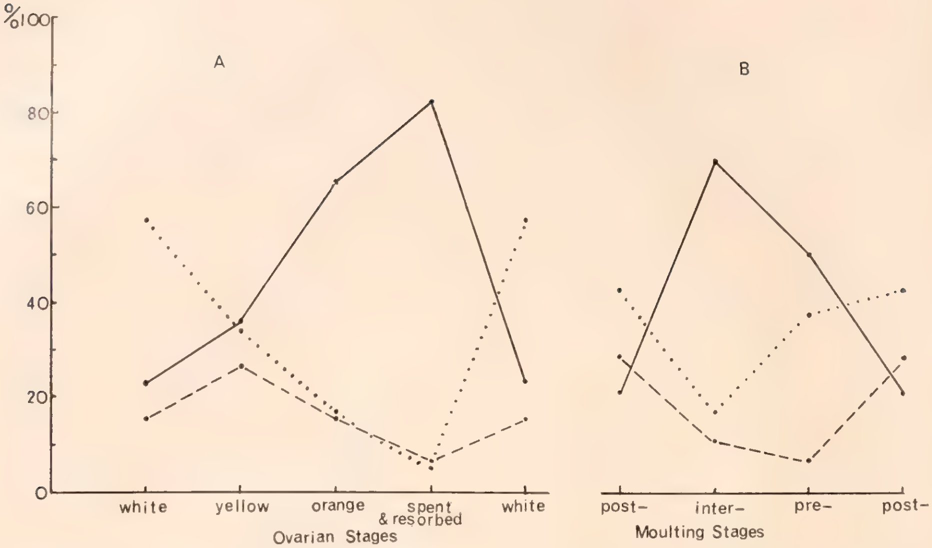

FIGURE 2. A. Ovarian stage and shell color. B. Molting stages and shell color. Postmolt, intermolt and premolt are here defined in the same way as in Figure 1. Green ......; yellow ------; orange-red ———.

aquarium, they usually acquired bright golden yellow, or less often, brownish color, on the underside. The reason for such different color-changing in the smaller crabs is not yet understood.

DISCUSSION

It was mentioned above that the present method of collection excluded nonfeeding crabs around molting time. If, then, those characteristics discussed here did not bias the sampling by trapping, then we may make the following conclusion.

The three cycles, ovarian maturation, molting, and color change, are apparently under different hormonal control in *Carcinus* since any combination of their stages could occur, though correlation between certain stages of the different cycles is found.

Such correlations support, in general, the conclusions of Démeusy and Lenel (1954), and of Démeusy (1963), that the ovarian and molting principles are antagonistic to each other in *Carcinus*, that egg-laying takes place when the crabs are at their intermolt stage, and that molting occurs mostly after the spent stage. The present observations show that, in nature, this antagonism may not always be effective. For example, a molting crab with full-size mature orange ovary was recorded twice during the study.

The color in the shell is undoubtedly the result of biochemical transformations during the metabolism of carotenoids, as indicated by previous workers (Lenel and Veillet, 1951, etc.). These authors have demonstrated that removal of the eyestalks from freshly molted *Carcinus* resulted in a marked reddening, affecting the new cuticle as well as the epidermal chromatophores. According to them, this reddening is due to the dissociation of the brown and green astaxanthin-protein complexes. This would probably explain the formation of the orange-red color in the present study (see Goodwin, 1960, for review of the subject).

It is difficult to assign any advantage to color changes during the molting and ovarian-developing cycles. However, there is known to be some habitat change during the cycle.

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SUMMARY

1. The three decapod characters, molting, ovarian developing and shell color changing, known to be under hormonal control were studied in the female shore crab, *Carcinus maenas* (L.), by the sampling method.

2. Results indicate that (a) the above three cycles do not appear to be under the same hormonal control; (b) a negative correlation exists between molting and ovarian maturation. This confirms, in general, experimental results of previous authors that there is an antagonism between molting and ovarian maturation. On the other hand, this antagonism does not seem to be always effective in nature. (c) A contrasting occurrence of green and orange-red shell color is established in the molting as well as ovarian maturation cycles. (d) The significance of the shell color changes is discussed.

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A QUANTITATIVE EXAMINATION OF OVARIAN GROWTH IN THE WHITE-CROWNED SPARROW[1]

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In an earlier paper in this journal (Farner and Wilson, 1957) it was demonstrated that testicular growth in *Zonotrichia leucophrys gambelii* induced by stimulatory daily photoperiods of constant duration is approximately a logarithmic function of time from resting testicular weight until a weight of 200–250 mg. is attained. It was demonstrated further that the slope represented by the logarithmic growth-rate constant (k) has an approximately linear relationship to the duration of the daily photoperiod (p) over a wide range of values (*ca.* 9 to 18 hours) of p. Subsequently, a much greater accumulation of unpublished data has confirmed these relationships. Similar relationships have been demonstrated for *Chloris chloris, Fringilla montifringilla,* and *Fringilla coelebs* by Dolnik (1963), and apparently exist also in *Passer domesticus* (Bartholomew, 1949; Farner and Wilson, 1957; Farner, 1964; Vaugien, 1952, 1954, 1959; Middleton, 1965) and in *Coturnix coturnix japonica* (Mather and Wilson, 1964; Tanaka *et al.*, 1965; Follett and Farner, unpublished), although, as yet, the precise functional relationships of k to p are not known for the latter two species.

At the time of our investigation of the quantitative relationships between p and k in testicular growth in *Zonotrichia leucophrys gambelii,* we became aware of a basic photoperiodic control in the annual ovarian cycle. The much slower development of our knowledge of the role of the photoperiodic mechanism in the control of the ovarian cycle involves several factors. Prominent among these is the obviously slower and more variable growth rate of the ovary under photoperiodic stimulation. Because of this it has always been essential to use males in photoperiodic experiments in which quantitative data on response rates are essential. Also, contrary to the situation in males, complete gonadal development does not occur in captive females, regardless of photoperiodic regime (Farner, 1959, 1964). Furthermore, the accumulation of data on the ovarian cycle, both in the field and in the laboratory, has been necessarily at a much slower rate because of the very low fraction of females in the wintering flocks in the Snake River Canyon (Mewaldt

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and Farner, 1953), a major source of our experimental birds. However, we have now accumulated sufficient data to permit some quantitative characterization of photoperiodically induced ovarian growth. It is the function of this paper to present these data, together with our analyses and interpretations thereof.

We wish to acknowledge the generous assistance and advice of Professor Brina Kessel of the Department of Biological Sciences and Professor Laurence Irving of the Laboratory of Zoophysiology, University of Alaska, in conjunction with our field investigations in the vicinity of Fairbanks. Standard preparations of gonadotropin were kindly supplied by the Endocrinology Study Section, National Institutes of Health.

Materials and Methods

The experimental birds were captured with mist nets from migrating flocks in the vicinity of Pullman in autumn, and from a wintering population in the nearby Snake River Canyon. They were held in large outdoor aviaries until the end of January. At that time they were moved indoors and placed two or three per cage into small cages (41 × 22 × 26 cm.) on 8-hour daily photoperiods (8L 16D). Light was provided by incandescent lamps at an intensity of at least 400 lux. Room temperature was maintained at 18–19° C. Food, consisting of a vitamin- and mineral-enriched chick-starter mash, and water were freely available at all times. Each bird was weighed weekly, at the same time of day, to the nearest 0.1 g. Sex was determined by laparotomy; only first-year females were used in the experiment.

Six weeks after the birds were brought indoors, 10 were killed to obtain resting ovary weights. Of the remaining birds, 10 were retained as controls on 8L 16D and 50 were placed on 20-hour daily photoperiods (20L 4D) by extending the termination of the photoperiod by one 12-hour increment. The latter group was sampled at selected intervals during the next seven weeks, at the end of which time all remaining birds were killed.

Ovaries were fixed in a solution of 10 parts acetic acid, 10 parts formalin, 30 parts 95% ethanol, and 50 parts water for five days and then transferred to 70% ethanol for five additional days. They were then carefully debrided of extraneous tissues and weighed on a torsion balance. Anterior pituitary glands were removed immediately after killing and placed in dry acetone. The acetone was changed twice during the ensuing four days, after which the glands were air-dried and then stored *in vacuo* over phosphorus pentoxide at 0° C.

For interpretation of our laboratory data with respect to the natural ovarian cycle we have used data on ovarian weights from birds taken in the field in winter in the Snake River Canyon, during spring migration in the vicinity of Pullman, and during the breeding season in the vicinity of Fairbanks, Alaska. These birds were obtained by shooting or with mist nets. Treatment of the ovaries was similar to that for the experimental birds.

Gonadotropin was assayed by a modification of the method of Breneman *et al.* (1962), the details of which are being published elsewhere. In essence, day-old chicks were injected subcutaneously with either a homogenized pituitary extract from white-crowned sparrows or with a standard LH preparation (NIH-LH-S7);

six hours thereafter they received a dose (1.2 μc.) of carrier-free P^{32}. Eighteen hours later all animals were killed; the testes were removed, blotted and weighed to the nearest 0.1 mg. They were dried on aluminum planchets and counted with a Nuclear-Chicago gas-flow counter (Model D47) with automatic sample changer. Results were expressed as counts (minus background) per minute per mg. wet tissue.

Estimates were made with a balanced four-point factorial assay method. Eight to 10 chicks were used at each dose level. The results from an experiment were subjected to a full analysis of variance (Bliss, 1952); potency estimates are given, together with 95% confidence limits and the index of precision (λ). The latter shows the method to compare favorably with other methods of gonadotropin assay. A standard preparation of LH was employed since it allowed the estimate of potency from a single assay. In all cases glands from a number of birds were pooled for one assay; however, the method could be used to measure the gonadotropic content of individual glands.

OVARIAN WEIGHT AS A FUNCTION OF TIME WITH A CONSTANT DAILY PHOTOPERIOD OF STIMULATORY DURATION

An examination of the weights of the ovaries from the photostimulated group (Fig. 1) suggests that there is an approximately linear relationship between the logarithm of ovarian weight and time that may be expressed as

$$\log_{10} W_t = \log_{10} W_0 + kt,$$

where W_0 is ovarian weight in milligrams at the beginning of treatment with 20L 4D, W_t is the weight at t days, t is time in days on 20L 4D, and k is the logarithmic growth-rate constant in days^{-1}. It should be noted that k for ovarian growth in this experiment, 0.027 ± 0.003 days^{-1}, is much smaller than that, 0.111 ± 0.011, for a group of first-year males subjected to the same photoperiodic treatment at approximately the same time. This difference in laboratory-induced rates between the sexes is consistent with the generally observed slower rate of ovarian development under natural conditions in early spring (Table I) in which the increase in weight by the time of spring migration is approximately four-fold, compared with a 50-fold increase in testicular weight. If, as there is some reason to assume, the steady-state level of pituitary gonadotropin has a positive functional relationship to the rate of gonadotropin output, one must tentatively conclude that a given rate of output causes a more rapid testicular growth than ovarian growth (Table I). However, we cannot exclude the possibility that the relationship between pituitary gonadotropin level and the rate of output may be different in the two sexes.

The failure of ovarian growth to proceed beyond approximately 50 mg. in the birds subjected to 20L 4D is consistent with our long, although somewhat casual, experience with the behavior of females in captivity. In the 15 years in which we have used this species experimentally, only twice have we found single eggs in the outdoor aviaries; the production of eggs has been equally infrequent by females held in cages indoors (Merkel, 1963). Of interest here are a series of observations on levels of pituitary gonadotropins and gonadal development in spring in wild birds and birds held in large outdoor aviaries under

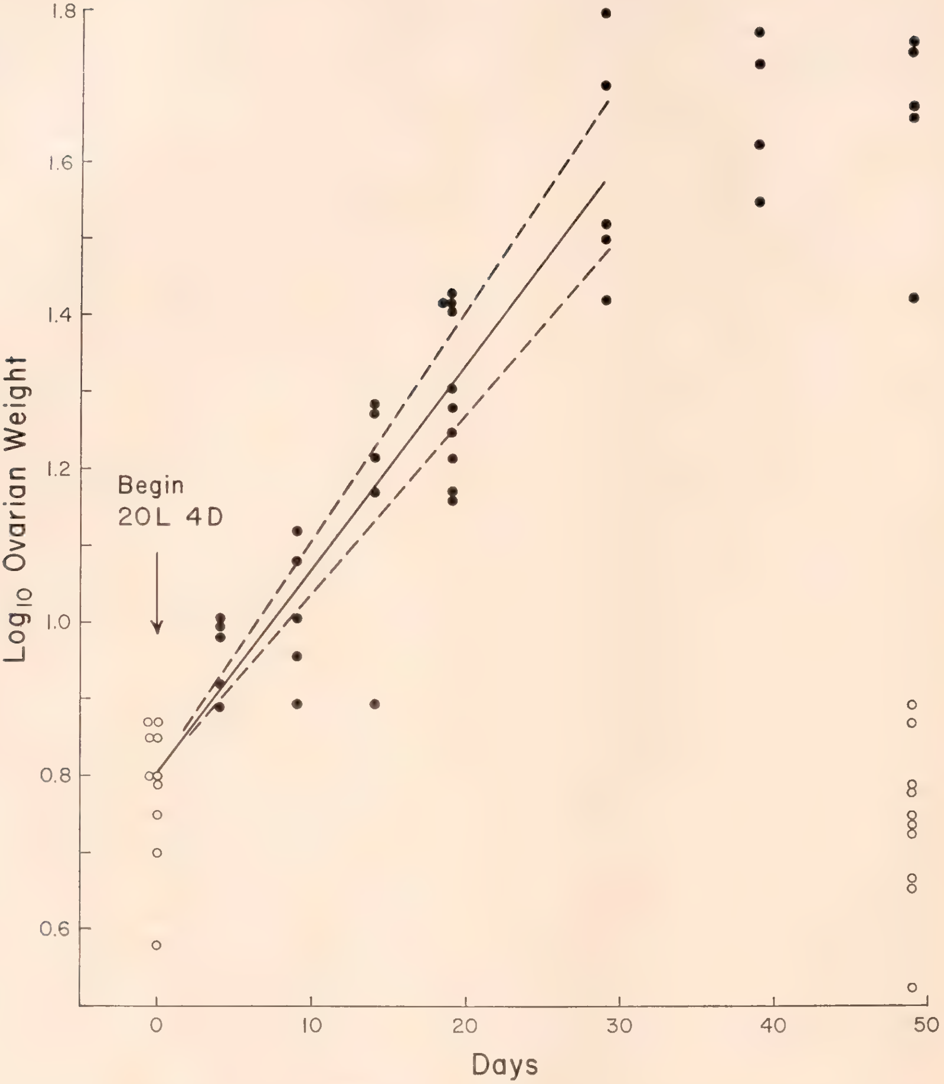

FIGURE 1. Ovarian weights (*W*) of first-year white-crowned sparrows as a function of time after a change in daily photoperiod from 8 (8L 16D) to 20 hours (20L 4D). Closed circles represent birds subjected to 20L 4D; open circles represent birds continued on 8L 16D.

natural conditions of temperature and photoperiod (Table I; also King *et al.*, in press). Migrating females taken in Pullman in early May had a mean ovarian weight of 18.6 ± 1.5 mg. and a pituitary gonadotropin content of 11.1 (8.4–14.7) μg./gland. For females taken late in May, shortly after arrival on the breeding grounds in the vicinity of Fairbanks, Alaska, the respective means were 326 ± 129 mg. and 9.8 (7.5–12.1) μg./gland. In contrast, the respective means

TABLE I

Relationship between gonadotropic potency of the anterior pituitary and relative gonadal weight (W) in male and female Zonotrichia leucophrys gambelii under natural conditions

Period	Male			Female		
	W* mg.	W/W_{max}	Pituitary gonadotropin**	W* mg.	W/W_{max}	Pituitary gonadotropin**
Mid-winter	1.1 ± 0.1	0.002	3.0 (2.6–3.6)	4.9 ± 0.5	0.015	3.1 (2.3–4.1)
Spring migration	47.7 ± 4.1	0.108	11.1 (9.4–14.1)	18.6 ± 1.5	0.057	11.1 (8.4–14.7)
Breeding ($W_{maximum}$)	442. ± 31.	1.000	14.8 (13.5–16.1)	326. ± 86.	1.000	9.8 (7.5–12.1)

* Mean and standard error.

** Microgram equivalents NIH-LH per gland; means with 95% confidence limits.

for females captured during spring migration and held in outdoor aviaries in Pullman until 3 June were 30.2 ± 2.8 mg. and 2.7 (1.6–4.4) μg./gland. The situation in the males was very different. For the spring migrants in Pullman the mean testicular weight was 47.7 ± 4.1 mg. and the pituitary gonadotropin 11.1 (9.4–14.1) μg./gland. The respective means for the breeding males taken in late May were 408 ± 14 mg. and 14.8 (13.5–16.1) μg./gland. However, males held in outdoor aviaries in Pullman until the first week of June showed only slight differences from the breeding sample, the means being 353 ± 16 mg. and 16.9 (12.6–22.8) μg./gland. Consistent with the observations of an approximately 50-mg. limit on ovarian growth in photoperiodic experiments are our unpublished observations of the failure of captive females to develop the brood patches and hypercalcemia that characterize the egg-laying period.

Consideration of the initial logarithmic growth of the ovary of birds subjected to long daily photoperiods, and of the behavior of the gonadotropic activity of the anterior pituitary in the experiment described herein and in unpublished experiments, leads to the conclusion that the gonadotropic function of the anterior pituitary in photostimulated females, until the 50-mg. ovary is attained, is essentially the same as in photostimulated males. The intriguing problem, of course, is the great difference between the sexes beyond this point. There is the possibility that, for reasons as yet unknown, the control system, in captive females, fails to maintain the level of gonadotropin release characteristic of the initial period of ovarian growth. There is also the possibility that growth and development of the ovary beyond the 50-mg. level requires a second gonadotropin which is not produced in captive birds. This can only be resolved by further investigations.

Equally interesting is the problem of the environmental etiology of the failure of the ovaries of captive females to develop beyond the 50-mg. level. Although we have no observations on which to base the suggestion, there is the possibility of some critical deficiency in the environment, such as Polikarpova (1940) was able to conclude from his experiments with *Passer domesticus*. In this species it appears, as in *Zonotrichia leucophrys gambelii*, that photoperiodic stimulation can cause the development of the ovary only up to a certain stage of development; further development comes only in the presence of an active male, and complete development occurs only if nesting sites and nesting material are available. It is possible that in *Z. l. gambelii* the females have similar requirements, although our data suggest that active males do not exert such an influence on females in captivity. Whether or not active males in the migrating flocks, during the approximately three weeks that elapse between departure from the latitude of Pullman and arrival in the breeding areas in Alaska, can account for the difference in ovarian development between wild and captive birds is conjectural. Because of the partial separation of males and females in spring migration (King *et al.*, 1965) we suspect that an additional or alternate factor must be involved. It is possible also that conditions of captivity impose an inhibitory "fear reaction," such as that described by Phillips and van Tienhoven (1960) for female mallards (*Anas platyrhynchos*) and female pintails (*A. acuta*). Electrolytic lesions in the ventral medial archistriatum or occipito-mesencephalic tract resulted in the disappearance of "escape behavior" and in the development of the ovary (Phillips, 1964). Again it appears unlikely that such an explanation can be entirely adequate for *Z. l. gambelii* since development of the ovary up to the 50-mg. level proceeds normally under photoperiodic stimulation in caged birds. It is obvious that much additional research lies ahead in order to identify the environmental deficiency or inhibitor imposed by captivity on the females of *Z. l. gambelii*.

NON–PHOTOPERIODIC OVARIAN GROWTH

An examination of ovarian weight in the first-year birds reveals, from the post-nestling stage until midwinter, an approximately linear increase (Fig. 2) that appears to be independent of the natural daily photoperiod. This conclusion is sustained by the observation of continued growth when birds are transferred to 8L 16D in midwinter to avoid the increasing natural daylength and subsequent photoperiodically induced ovarian growth. The regression line for all birds is shown in Figure 2; the equation for the line, based on 191 ovarian weights, is $W = 1.067 + 0.0186t$. Statistically, this slope is significantly greater than zero ($P \ll 0.001$).

An analysis of the ovarian weights for 76 adults indicates a similar but much smaller non-photoperiodic growth, the regression equation being $W = 4.693 + 0.0062t$. The slope of the line is of marginal significance ($P \lessgtr 0.05$); however, the difference in slope between first-year females and the adults is highly significant ($P \ll 0.001$).

Little further can be said about this non-photoperiodic ovarian growth at this time. It would be of interest to learn how long it can continue under a regimen of short daily photoperiods. Insofar as the overall development of the ovary is concerned, the quantitative contribution of the non-photoperiodic phase is slight. Even first-year birds, at the rate shown in Figure 2, would require eight years, or about 5 times the mean life span, to attain the 50-mg. ovary characteristic of the migrant at the latitude of Pullman. This re-emphasizes the importance of the duration of the daily photoperiod in the basic timing of the ovarian cycle. Although the magnitude of the non-photoperiodic ovarian growth is small, it is important that it be recognized in photoperiodic experiments since serious errors can be introduced in the calculation of the logarithmic growth-rate constant by

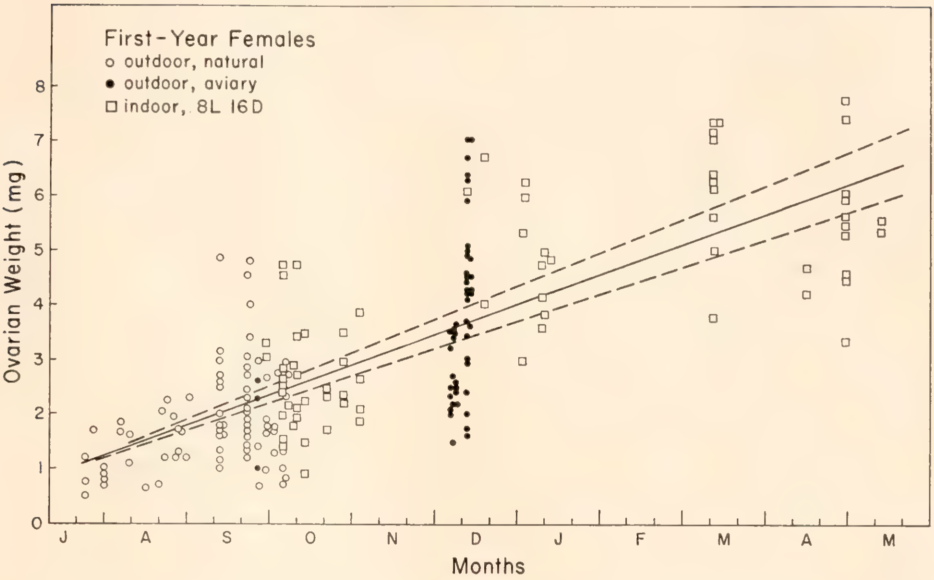

FIGURE 2. Non-photoperiodic growth of the ovary of the first-year white-crowned sparrow (*Zonotrichia leucophrys gambelii*). Ovarian weight is plotted as a function of time in months; during this period the birds did not receive stimulatory photoperiods. ○, birds taken from the field; ●, birds from outdoor aviaries under natural conditions of temperature and photoperiod; □, birds held indoors under 8L 16D. Field and outdoor aviary birds are not included beyond mid-January since natural photostimulation can first be detected in February.

failure to have a precisely determined W_0 for the date of initiation of long-day treatment.

Inspection of our data on testicular weights for first-year males has frequently prompted us to suspect the occurrence of a very slight non-photoperiodic growth through late fall and early winter. Despite the much larger amount of data available, our statistical analyses fail to sustain our suspicion. Certainly if such growth does occur, it is extremely slight. We find in our data from several hundred adult males no reason to suspect a non-photoperiodic testicular growth.

SUMMARY

1. Ovarian development in *Zonotrichia leucophrys gambelii* subjected to long daily photoperiods is a logarithmic function of time until a weight of approximately 50 mg. is attained. Although there is a slight non-photoperiodic ovarian growth, especially in first-year birds, it is clear that long daily photoperiods are essential for normal ovarian growth and constitute the basic environmental information used in the control of the ovarian cycle.

2. The photoperiodic control mechanism in the female differs from that of the male in that the logarithmic growth phase brings the ovary to only approximately one-tenth of its maximum weight, whereas the logarithmic growth phase of the testes in photostimulated males brings them to about one half of maximum

TABLE II

*Pituitary gonadotropin content in juvenile female Zonotrichia leucophrys gambelii exposed to long daily photoperiods**

Day of treatment	Mean gonadotropic** potency	95% Confidence limits	Lambda
0	7.9	5.8–10.3	0.157
4	5.4	3.8– 6.7	0.108
9	12.7	10.0–15.6	0.122
14	15.2	12.2–18.7	0.119
19	15.9	11.7–21.9	0.167
29	13.1	7.8–17.6	0.168
49	14.2	10.6–18.7	0.155
49 (final 8L 16D controls)	6.6	4.8– 8.2	0.116

* Twenty hours per day (20L 4D), after being held previously on short days (8L 16D).

** Microgram equivalents NIH-LH per gland; means with 95% confidence limits.

weight. In addition, the female differs from the male in that complete gonadal development can be induced only rarely in captivity with either artificial or natural photoperiodic stimulation, whereas sufficiently long daily photoperiods invariably result in the complete development of the testes of photosensitive males in captivity.

3. Until an ovarian weight of approximately 50 mg. is attained the gonadotropic performance of the anterior pituitary of the female in captivity appears to be similar to that of photostimulated males.

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REPRODUCTION AND EARLY DEVELOPMENT OF THE SPIONID POLYCHAETE, SCOLECOLEPIDES VIRIDIS (VERRILL)

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As far as is known the sedentary polychaete, *Scolecolepides viridis* (Verrill), is confined to the east coast of North America where it is found from Newfoundland to South Carolina (Pettibone, personal communication). The worm is most common intertidally but occurs down to 20 fathoms. It burrows in a variety of substrata and is particularly abundant in sand (Verrill, 1873; Webster, 1879; Webster and Benedict, 1884; Wells and Gray, 1964). The worm is most frequently found in areas of reduced salinity (Cowles, 1931; Stickney, 1959; Smith, 1964). The adults inhabit vertical mucus-lined burrows and feed on the surface deposit aided by a pair of ciliated tentacles that draw food into the region of the mouth where it is engulfed by eversion of the pharynx. Sanders *et al.* (1962) found the gut contents of specimens from Barnstable Harbor, Massachusetts, to consist of sand, detritus, diatoms, filamentous algae, and a nematode.

Little information is available regarding the reproduction and early development. Dr. David Dean (personal communciation) found specimens with eggs and sperm in February and March in the Mystic Estuary, Connecticut, and Dr. Marian Pettibone (personal communication) noted worms massed with eggs in the Oyster River, New Hampshire, at the beginning of April. Dr. J. P. Moore (unpublished manuscript) reported that breeding occurred in the Woods Hole region in July and August. Mead (1879) followed the first few divisions of an egg of a worm which he called *Scolecolepis viridis* but which, in my opinion, was wrongly attributed to this species.

MATERIALS AND METHODS

The population chosen for the study is located in intertidal mud-sand at Lawrencetown, Nova Scotia. The worms are living in estuarine conditions, often experiencing considerable changes in salinity during a tidal cycle (1–32‰). The worms are not distributed evenly over the flat but show marked aggregation (maximum density of adults—1000 per m.^{2}) which cannot be correlated with particle size of the substratum or with its organic content.

As the sole description of the species (Verrill, 1873) is brief and unillustrated it is necessary to include at this point a redescription of the worm before considering its early development. The adult worm is composed of up to 250 segments and has an approximate length of 140 mm. and a width of 3 mm. It may be colored various shades of green or brown but invariably has red branchiae.

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The body, which in cross-section is convex dorsally and flattened ventrally, is widest at the anterior end and tapers posteriorly. The prostomium, whose anterior end forms distinct frontal horns, narrows posteriorly and usually bears two pairs of eyes (often only one pair or none) (Fig. 1A). The grooved tentacular palps arising from the peristomium are short and stout and spotted with black pigment. A ciliated vestibule bounded laterally by thick lips leads to the gut (Fig. 1B). The bilobed parapodia begin on the first post-peristomial segment. All parapodia bear short capillary setae (Fig. 1E) with spreading bundles of long setae at the dorsal and ventral extremities (Fig. 1C, D). The hooded

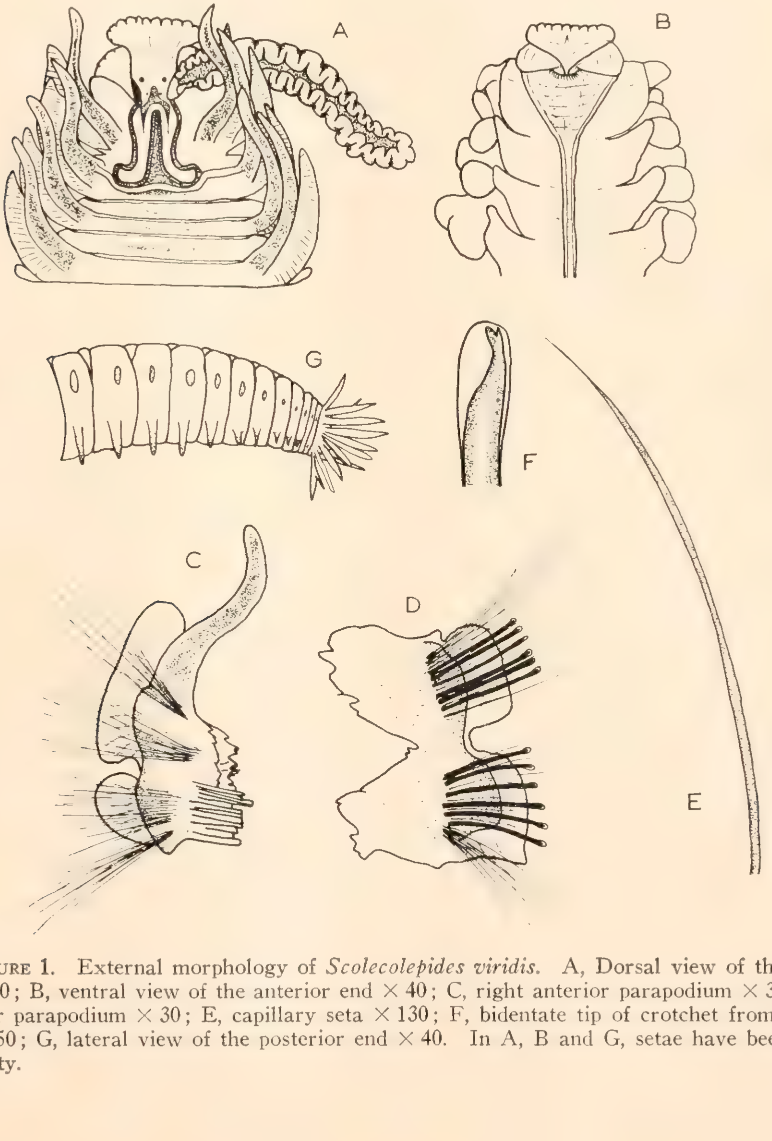


FIGURE 1. External morphology of *Scolecolepides viridis.* A, Dorsal view of the anterior end × 40; B, ventral view of the anterior end × 40; C, right anterior parapodium × 30; D, left posterior parapodium × 30; E, capillary seta × 130; F, bidentate tip of crotchet from posterior end × 250; G, lateral view of the posterior end × 40. In A, B and G, setae have been omitted for clarity.

bidentate crotchets (Fig. 1F) are absent from the anterior setigers, first appearing in the neuropodium between segments 20–50 and in the notopodium between segments 35–65. There are up to 11 crotchets per lobe. Immediately behind the bristle bundles on each segment are neuropodial and notopodial post-setal lamellae, which become less prominent more posteriorly. The ligulate branchiae, of which there is one pair per segment, begin on the first setiger. They are prominent in the anterior third of the body where they are fused to the post-setal lamellae. The branchiae become progressively smaller towards the middle of the body where they eventually disappear. The posterior end is truncate and the terminal anus is surrounded by cirri (8–20) which are sometimes dichotomously branched (Fig. 1G). The worm has ciliated bands intersegmentally and also a band in the middle of each segment which continues laterally on to the parapodia. On the dorsum of the first two segments there is a pair of ciliated epaulettes (Fig. 1A). The ciliation of the dorsum and branchiae is stronger than that of the rest of the body. These dorsal cilia are the main propulsive force behind the water currents aerating the burrow of the worm. Water is swept towards the posterior end along the tunnel formed by the arched branchiae. Over the rest of the body surface the cilia beat towards the anterior end. Occasionally individuals reverse the direction of the ciliary beat.

To obtain evidence for the onset of breeding, worms were collected at monthly intervals and examined for the presence of sexual products in the coelom. As the breeding season neared, weekly collections were made. On the high tide preceding or following the collection of the adults, three 500-liter pump samples were taken from the sea water overlying the flat. The water was filtered through 125-μ and 63-μ mesh nets, and examined for eggs and young larval stages of the worm.

Permanently installed maximum-minimum thermometers were used to record the air, mud, and sea water temperatures. Where possible a portable induction salinometer was used to determine the salinity, otherwise water samples were processed in the laboratory, using the Mohr method.

Adult worms were maintained in the laboratory in flat-bottomed glass culture dishes containing filtered sea water from the sampling site. The aerated sea water in the vessels was changed twice a week and normally kept at a temperature approximating that prevailing in the field. The worms were supplied with lengths of glass tubing which they used as "burrows." The majority of larval experimental cultures resulted from naturally spawned eggs and sperm, but occasionally eggs were fertilized using sperm cut from mature males. After fertilization eggs were transferred to clean sea water. Some were allowed to develop at 2°, 10° and 20° C. in 30‰ sea water, whilst others were kept at temperatures of 2° C. or 10° C. and allowed to develop at different salinities (5, 10, 15, 20 and 30‰).

Both adults and larvae were fed on a sea-water suspension of *Ulva* which had been dried and then finely powdered. Living developmental stages were photographed in watchglasses, the ciliated larvae being slowed when necessary by addition of $MgCl_2$ crystals to the sea water. The photographs were taken by transmitted light through a Leitz Orthrolux photomicrographic unit, using Panatomic X film and a red filter over the light source to increase the contrast.

Breeding Period

Seasonal variation in the percentage of worms containing genital products

Ripe males and gravid females were present in approximately equal numbers (Fig. 2). When sampling started in late February, 1963, 96% of the population contained gametes. During March this figure dropped to 11% and by the end of April only a few ripe males remained. In June, July, August and September no gamete-bearing animals were found. Gravid females appeared in October and by the latter part of November ripe males were also present, bringing the total percentage of worms with genital products to 70%. The percentage continued to rise and by the beginning of January, 1964, did not differ significantly from 100%. During January, February and March the percentage stayed at this high level, but the first week in April saw a marked decline to 12%. Thereafter, as in the previous year, only a few ripe males remained until the end of May. Sharp declines in the percentage of worms with genital products suggest that maximum breeding activity occurred during March in 1963 and at the end of March and beginning of April in 1964.

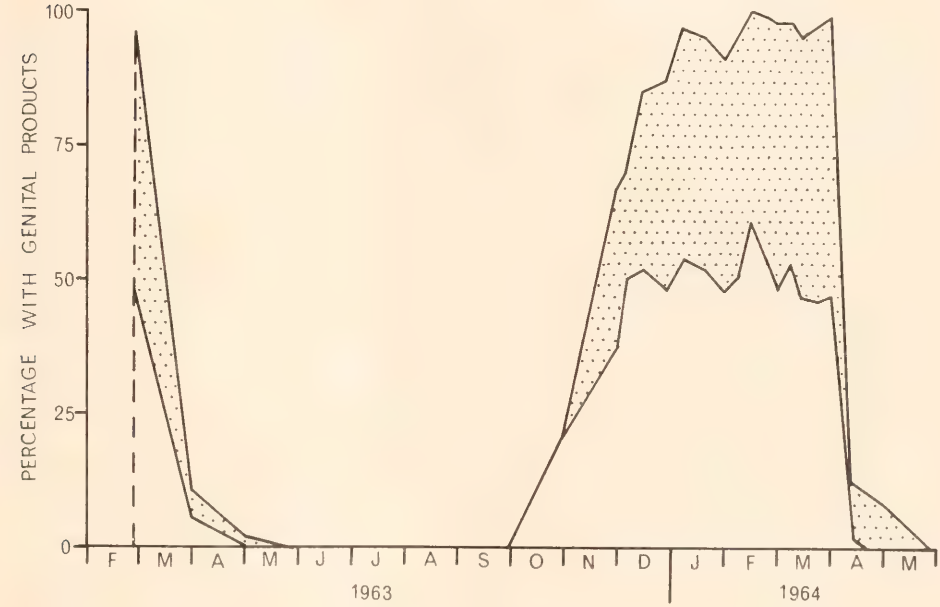


Figure 2. Seasonal variation in the percentage of worms containing genital products. Open area = females; stippled = males.

The data also reveal that the worms must reach maturity in less than one year, since all worms are gravid at one time.

Seasonal variation in the quantity of genital products in adult worms

Four categories, similar to those used by Joyner (1962), were adopted to record the quantity of gametes present in the reproductive region of the worms (see legend to Fig. 3).

In late February, 1963, the majority of the males were half-full with gametes and at least 25% had coeloms that were full (Fig. 3). A high percentage of the females (44%) were full. By the end of March all the females were spent individuals as were the majority of the males. It was not until the following October that gametes again appeared, and individuals collected in November and December were predominately those with gametes present only in the base of the parapodia. By the end of January, 1964, however, many were half-full with gametes. In February half-full worms predominated, as did full worms by the end of the following month, although several spent worms of both sexes were present by this time. Only spent animals could be found by the end of April.

The lack of full animals before February would indicate that breeding was not possible prior to this. In addition the Figure shows that all reproduction must have ceased by the end of March in 1963 and by the end of April in 1964. Fig-

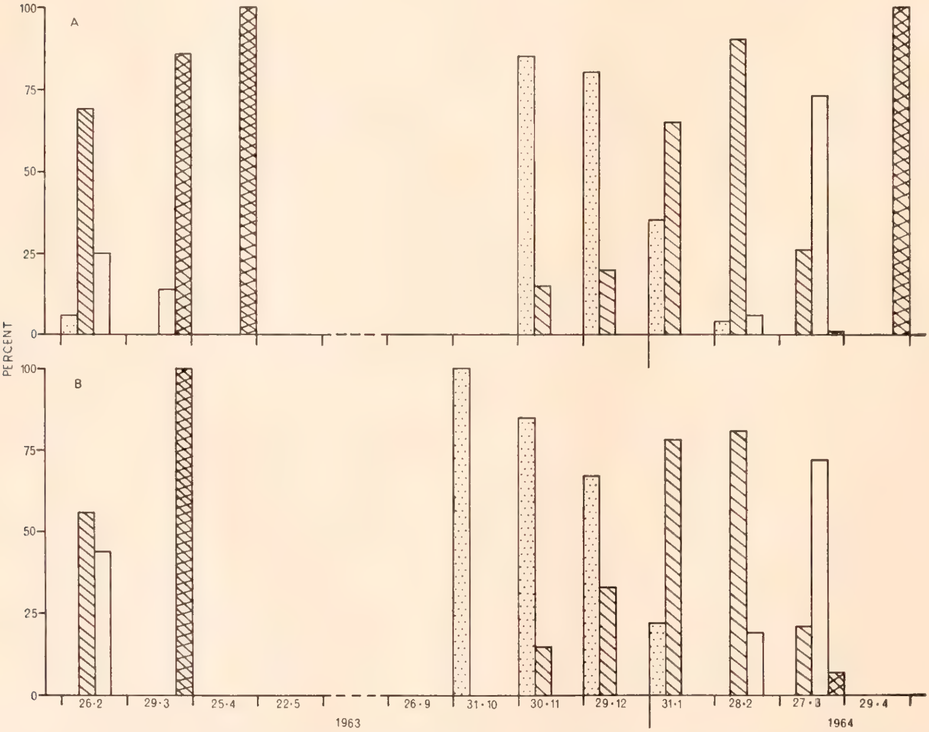


FIGURE 3. Seasonal variation in the quantity of genital products in the coelom of (A) males, and (B) females. Numbers in each category are expressed as a percentage of the total sample. Stippled bar = gametes in the base of the parapodia; bar with diagonal lines = coelom half-full; open bar = coelom full; cross-hatched bar = spent worms.

TABLE I

Number of eggs and larvae per m.^{3} *in the sea water overlying the sampling site. Data are means from three taken on each date.*

Stage of development	1963					1964									
	26.2	29.3	25.4	23.5	26.6	28.2	14.3	21.3	27.3	7.4	14.4	21.4	29.4	28.5	27.6
Unfertilized and dead oocytes	—	30	—	—	—	—	—	9	24,000	249	65	32	37	—	—
Early cleavage	—	350	—	—	—	—	—	5	80,750	510	32	6	13	2	—
Late cleavage and trochophore stages	—	20	—	—	—	—	—	—	—	162	2	-	2	—	—
1- to 3-setiger larvae	—	—	40	—	—	—	—	—	—	—	41	32	—	—	—
4- to 7-setiger larvae	—	—	—	—	—	—	—	—	—	—	3	2	12	2	—
8- to 11-setiger larvae	—	—	—	13	—	—	—	—	—	—	—	—	2	5	—

ures 2 and 3 both show that, initially, development of the male gametes lagged behind that of the female. This phenomenon was seemingly repeated at the end of the breeding period when ripe males were still present after all traces of gametes had disappeared in the females.

Presence of eggs and larvae in the plankton

At the end of March, 1963, eggs were found in the plankton, the majority of them being at early cleavage (Table I). Only 3-setiger larvae were present in late April but all had reached the 8- to 11-setiger stage by the end of May. Plankton samples taken in late June were free of young stages of *S. viridis.* The sea water overlying the sampling site remained clear until the end of March, 1964, when there was a large influx of eggs into the plankton. As at this time in the previous year the majority of the eggs were at early cleavage. By the end of the first week in April trochophores were present in the plankton. Throughout April the larvae increased in number of setigers until by the end of the month the larvaae possessed 4–11 setigers. A sample at the end of May showed few larvae remaining, the largest number being at the 8- to 11-setiger stage.

Although the scant data for 1963 show only that breeding was taking place during March, those for 1964 indicate that a very large spawning burst occurred at the end of March in this year. Numbers of animals obtained in the plankton samples are too low to draw any firm conclusions as to mortality and growth rate in the field, although the figures suggest that the larvae remain almost two months in the plankton, by which time they have reached the 10- to 11-setiger stage.

SPAWNING

The gonads (one pair/segment), which are attached to the nephridia, are absent only from the first 40–50 segments and the last 45–50 segments. During the breeding season the males and females can be readily distinguished by the color of their sexual products which are visible through the body wall. Sperm appear white and the oocytes orange/brown. The sperm are of the usual short-headed polychaete type (Franzén, 1956) with a pointed acrosome (Fig. 4). When mature, coelomic oocytes are disc-shaped with a diameter of 200–260 μ.

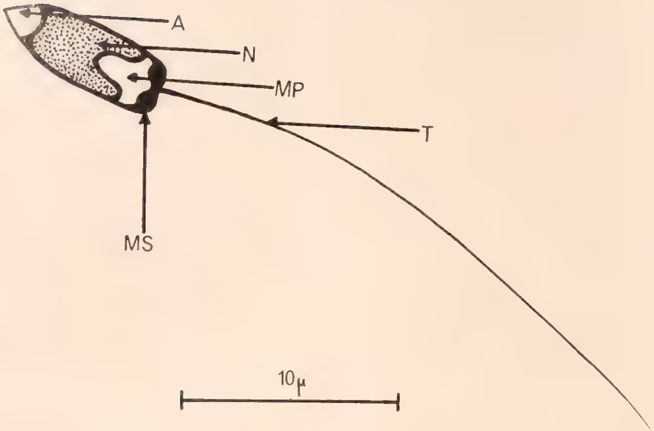


FIGURE 4. Spermatozoön of *S. viridis.* A = acrosome; N = nucleus; MP = middle portion; MS = mitochondrial spheres; T = tail. (Nomenclature according to Franzén, 1956.)

The egg-membrane is thick, the outer surface with a lattice-work of ridges giving it the appearance of a honeycomb, and the inner surface smooth (Fig. 5). Imbedded in the yolk round the periphery of the cell are 10–18 thin-walled vesicles connecting by narrow necks with small pores in the egg membrane. The nucleus and the membrane vesicles are translucent and colorless whilst the opaque yolk has a light green coloration in transmitted light. The number of oocytes produced by each female varies from 10,000 to 16,000.

Eggs and sperm are shed through mixonephridia (Goodrich, 1945) whose external openings are situated dorso-laterally and anterior to the notopodium. Spawning was not observed in the field but worms held in the laboratory shed their gametes whilst in their "glass burrows." The eggs and sperm were wafted out of the burrows in the currents set up by the cilia of the body surface. It is likely, therefore, that spawning in the field takes place in a similar fashion, especially as repeated excursions into the field by day and night failed to reveal any adults lying on the surface of the sand or swarming in the sea water over the sampling site. It must be noted, however, that the adults are good swimmers, moving through the water tail-first with a spiral motion.

Under controlled conditions in the laboratory, sudden salinity changes from 30‰ to 10‰ and from 10‰ to 30‰ led to spawning of 15% of the ripe females and of slightly fewer males (11%). Experiments in which animals were exposed to sudden temperature changes (within minutes) of 5° C. or 10° C. gave inconclusive results. It is clear that neither of the above factors is likely to be a major cause of spawning in the field population of *S. viridis.* No evidence was obtained to suggest that either sex was able to induce spawning in the other as a result of shedding, as is the case in many other invertebrates (Thorson, 1950).

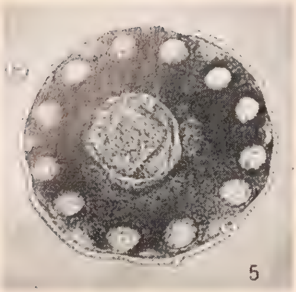
Early Development (At 10° C. and salinity of 30‰)

After fertilization, which could not be successfully accomplished in the laboratory below 5‰, the cytoplasm becomes detached from the inner side of the egg-membrane and starts to round off within an inner fertilization-membrane. The membrane-vesicles accompany the migrating cytoplasm and in so doing place their connection to the egg-membrane under tension, causing the egg-membrane to indent in the region of the pores (Fig. 6A). Simultaneously the disc-shaped egg takes on a more spherical form with the vesicles distributed equatorially (Fig. 6B). Following the initial contraction of the plasm, the membrane-vesicles increase in size and migrate from within the cytoplasmic mass, drawing tongues of material with them. The strain on the egg-membrane is thus relieved and the indentations disappear (Fig. 7). The tongues of cytoplasm retract into the central mass (Fig. 8), which rounds off, leaving the membrane-vesicles distributed between it and the egg wall (Fig. 9). This stage is reached in about 12 hours. As cleavage proceeds the vesicles become less visible and eventually disappear. The behavior of the vesicles is consistent with the function of volume-regulation ascribed to them in another spinoid worm by Hannerz (1956).

Cleavage is typical of the annelids, being unequal and spiral. The first cleavage produces a CD blastomere which is much larger than the AB blastomere (Fig. 10). At the 4-cell stage the D cell is readily distinguished by its large size (Fig. 11). After formation of the micromeres by a horizontal third cleavage (Fig. 12), division proceeds in a spiral fashion, making it almost impossible to follow in detail (Figs. 13 and 14). Gastrulation by epiboly occurs approximately two days after fertilization. The mass of cells now begins to take on a definite shape with a pointed front end (prostomium) from which apical cilia project through the egg-membrane (Fig. 15). As the prototroch differentiates, more cilia extend through the membrane and the embryo starts to rotate slowly, due to active beating of the cilia. This early larval stage can best be termed a pretrochophore since the ciliation is not yet strong enough to allow the larva to perform free-swimming movements.

The trochophore commences active swimming 3–4 days after fertilization and, unlike the adult, is strongly photopositive. Whilst swimming the larva slowly rotates about its longitudinal axis. The larva by this time has filled the space that existed between it and the egg-membrane and the membrane becomes incorporated as the larval cuticle. The crenulations that were so marked in the egg wall during cleavage stages are beginning to flatten out (Fig. 16). The prostomium has become rounded and the tuft of apical cilia more prominent. The prototroch girdle, which is formed on the raised area destined to be the peristomium, is composed of clumps of cilia which originate from well-defined pits in the body wall. Mid-dorsally there is a wide gap in the prototrochal ciliation. Ventrally the blastopore is readily visible. The region from which the gut will differentiate is seen as a dense mass of endoderm cells packed with yolk.

As the larva elongates the prostomium becomes dome-shaped and a pair of brown eyes, set wide apart, appears on it. A telotroch develops in the pygidial region and as in the prototroch the cilia occur in clumps with a mid-dorsal gap. The larval cuticle has by this time lost its sculpturing except at the posterior end. A small mouth leads into the yolk-filled gut in which three regions can be distinguished. Larval bristles on a raised area behind the prototroch mark the position of the first setiger (Fig. 17). The larva at this stage is approximately four days old.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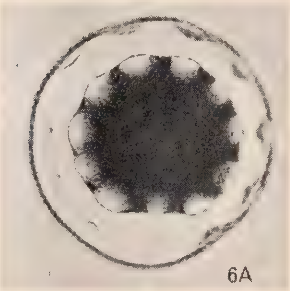
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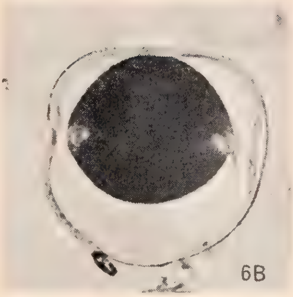
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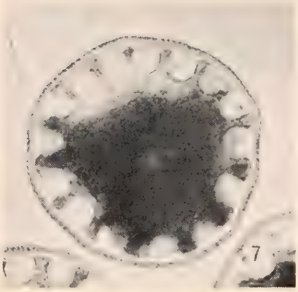
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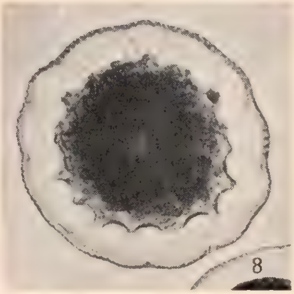
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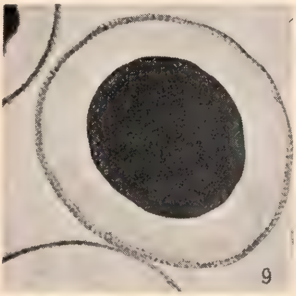
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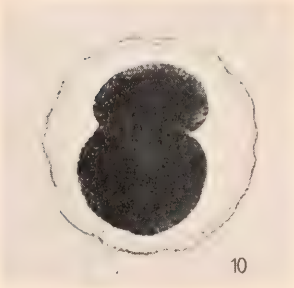
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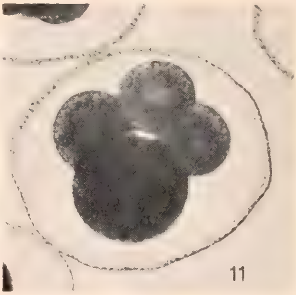
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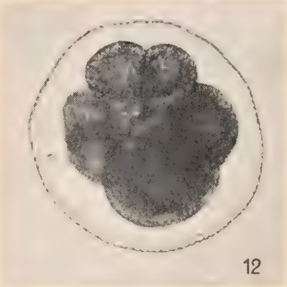
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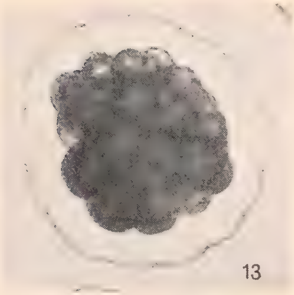
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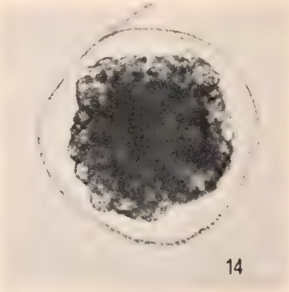
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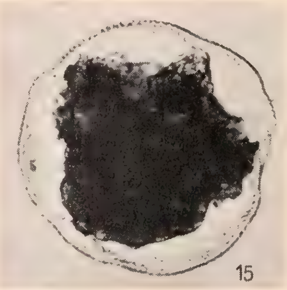
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FIGURE 5. Disc-shaped unfertilized egg.

FIGURE 6A. Fertilized egg. The cytoplasm has contracted away from the egg-membrane, drawing the membrane-vesicles with it. 6B. Side view of the same stage, showing the equatorial distribution of the vesicles.

By the time the pale green larva has reached the 3-setiger stage (Fig. 18) the prostomium has become flattened anteriorly and the long apical cilia have disappeared. At least three pairs of "sensory cilia" (*senso* Wilson, 1928) occur on the front edge of the prostomium and several are also found scattered on the underside of the prostomium and ventro-laterally on the pygidium. A second pair of eyes, smaller than the first, appears dorsally. The cilia of the prototroch have developed more strongly in the dorsal region, forming a continuous girdle broken only at the mid-dorsal gap. Ventrally the cilia of the prototroch are indistinguishable from the short cilia which are scattered over the concave ventral surface of the prostomium and peristomium. The cilia extend posteriorly as a neurotroch, ending in an inconspicuous ciliated pit on the second setiger. The slit-like ciliated vestibule is open anteriorly and ventrally and is bounded laterally by thick lips. The three regions of the gut are now fairly distinct. The mid-gut, which opens to the hind-gut through a narrow neck, is characterized by dark pigmentation. The few yolk granules that remain are confined to the mid-gut. Movement of food through the gut is facilitated by ciliation along its whole length and by periodic peristaltic waves starting at the anterior end. The larval setae are longest on the first segment and occur on the following segments in decreasing length. They are concentrated dorso-laterally in the notopodial position of each segment, and are finely serrated on their anterior margin.

The 3-setiger stage is reached in about 10 days but there is then a growth pause and the fourth setiger is not added for another 10 days. The pause may result in part from the final disappearance of the yolk granules and the larva having thus to rely entirely for its food supply on organic material removed from the sea water. It is at this stage that the greatest mortality occurred among larvae in the cultures. When once the larvae had passed this critical stage in their development mortality was much reduced.

As the larva develops beyond the 3-setiger stage the parapodia become more prominent, and a gastrotroch appears on the third segment. A smaller gastrotroch is formed on either side of the ciliated pit. By the time the larva reaches the 5-setiger stage (Fig. 19), it is approximately 425 μ long and 30 days old.

At the 9-setiger stage gastrotrochs are present on segments 3, 5, 7 and 9. Conspicuous nototrochs occur on segments 2, 3, 4 and 5, and smaller dorsal bands of cilia on segments 6, 7, 8 and 9. The notopodial (including branchial) and neuropodial regions of the parapodia have differentiated and small unserrated adult setae are beginning to appear. The tentacular palps are noticeable as small protuberances on the peristomium.

The larvae start to metamorphose at the 9- to 10-setiger stage. They be-

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FIGURE 7. Membrane-vesicles migrating from within the cytoplasmic mass, drawing tongues of material with them.

FIGURE 8. Tongues of cytoplasm retracting into the central mass.

FIGURE 9. Membrane-vesicles distributed between the egg-membrane and the rounded central mass of cytoplasm.

FIGURE 10. Two-cell stage, showing the AB blastomere and the larger CD blastomere.

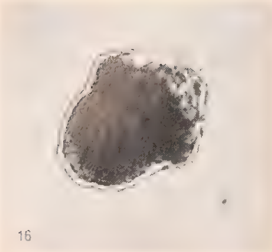
FIGURE 11. Four-cell stage with the large D cell clearly visible.

FIGURE 12. Eight-cell stage, showing formation of the micromeres.

FIGURE 13. Mid-cleavage with macromeres still visible.

FIGURE 14. Late cleavage.

FIGURE 15. Pre-trochophore with apical cilia protruding through the egg-membrane.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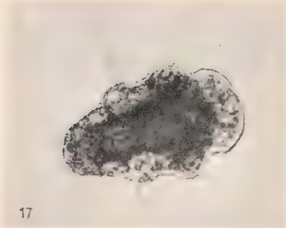
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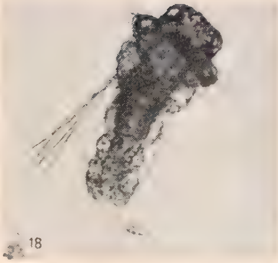
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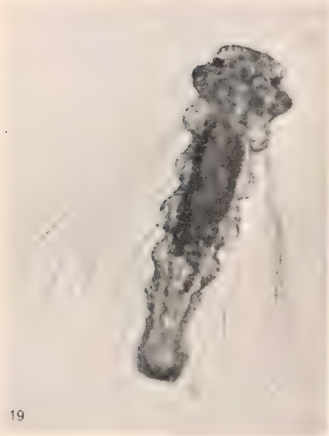
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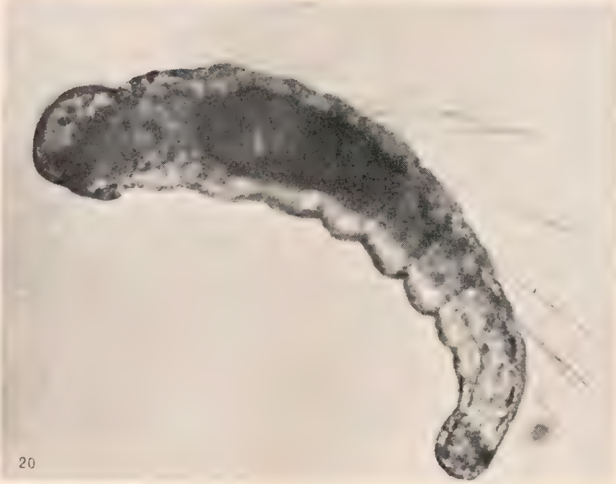
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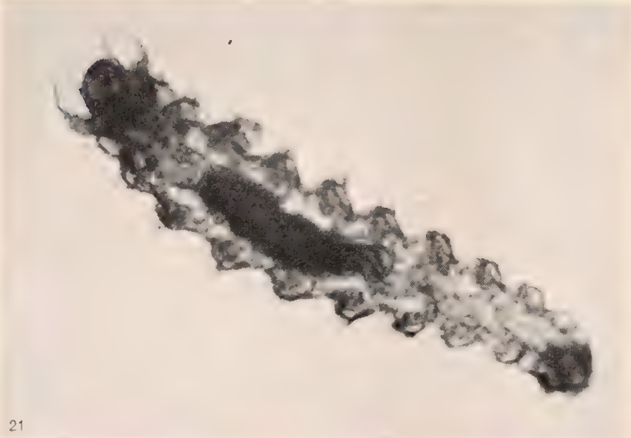
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Figure 16. Ventral view of a trochophore with the blastopore still visible.
Figure 17. Ventral view of 1-setiger larva.
Figure 18. Dorsal view of 3-setiger larva.
Figure 19. Dorsal view of 5-setiger larva.
Figure 20. Side view of 9-setiger larva.
Figure 21. Dorsal view of a recently metamorphosed individual with 10 setigers.

come photonegative in their reactions, and continually test the substratum for its suitability for burrowing, apparently using the "sensory cilia" of the prostomium. When no suitable substratum is available the larvae are able to postpone metamorphosis and will reach the 13-setiger stage, and then, in many cases, metamorphose successfully even in clean glass dishes. Many larvae begin to lose their larval bristles before settling but the majority are lost as the worms first burrow in the substratum. As noted by Dean (1965) for *Streblospio benedicti,* metamorphosis accelerates the development of the tentacular palps and branchiae (compare Figure 20 of a 9-setiger larva and Figure 21 of a recently metamorphosed individual). Adult setae also rapidly develop in both notopodium and neuropodium. The prototroch, gastrotrochs, neurotroch, ciliated pit and telotroch disappear during metamorphosis, although the nototrochs and oral cilia persist into adult life. The fully-metamorphosed 10-setiger worm is 750 μ long and approximately 40 days old.

As the young worms grow by addition of more segments, crotchets appear first in the neuropodium and then in the notopodium. Anal cirri become visible round the terminal anus, and the now-prominent branchiae become ciliated. By this time the young worm is readily recognizable as a young *S. viridis* and has adopted a position in a vertical burrow similar to that of the adult.

Effect of temperature on early development (At 30‰)

It can be seen in Figure 22 that sea water temperature affects the rate of growth. In laboratory cultures kept between 2° C. and 20° C. the growth rate increased with temperature until the 3-setiger stage. At 10° C. and 20° C. development continued after a growth pause, and metamorphosis was reached approximately twice as fast at 20° C. as at 10° C. At 2° C., however, growth virtually ceased when the 3-setiger stage was reached and in no cultures did the larvae proceed beyond the 3- to 4-setiger stage. That this is a temperature effect was demonstrated by increasing the temperature of the sea water in the cultures halted at the 3-setiger stage from 2° C. to 10° C. Within two days development was resumed and proceeded normally.

At 2° C. after prolonged periods (four weeks) at the 3-setiger stage many larvae lost their bristles, prototroch, and telotroch and sank to the bottom of the culture vessels, achieving what appeared to be a partial metamorphosis.

The upper temperature limit at which normal growth could take place was not accurately determined. However, temperature tolerance experiments at 30‰ showed that eggs and larvae, like the adults, were able to live for prolonged periods at 30° C. without any serious curtailment of growth. Death occurred between 34° C. and 35° C. The lower limit of temperature at which growth occurred was difficult to determine. Eggs and larvae could be supercooled at least to −5° C. without any harmful effects although none of the specimens appeared able to tolerate the formation of ice crystals in their bodies (*cf.* Kanwisher, 1955). Growth proceeded slowly up to the 3-setiger stage even in specimens held in sea water at −1.5° to −1.7° C.

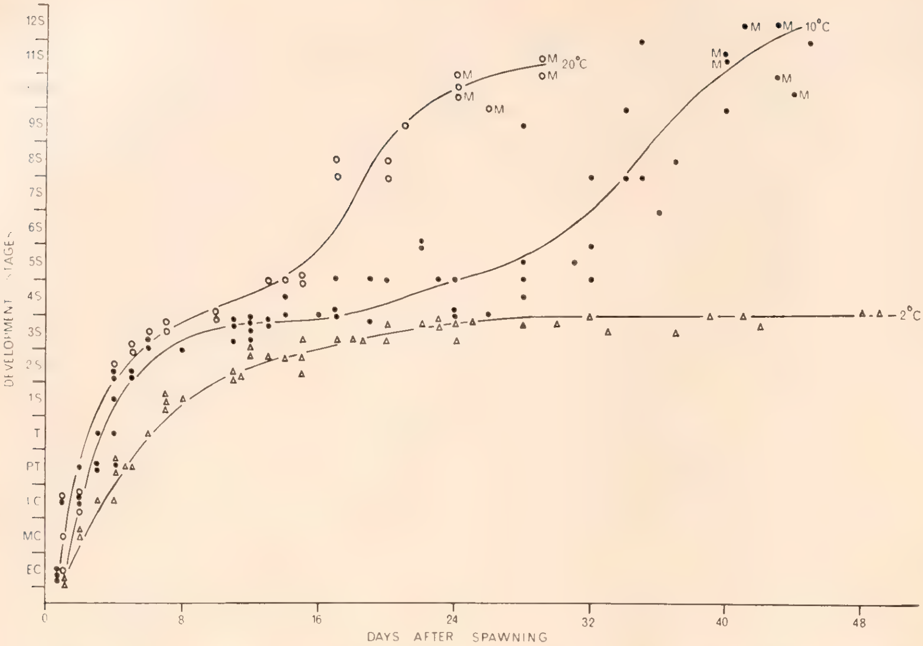

FIGURE 22. The effect of sea-water temperature upon the early development of *S. viridis* at 30‰. M = metamorphosed individuals; EC = early cleavage; MC = mid-cleavage; LC = late cleavage; PT = pre-trochophore; T = trochopore; S = setiger stage. Open triangles = 2° C.; Solid circles = 10° C.; Open circles = 20° C.

Effect of salinity on early development (at 2° C. and 10° C.)

There was no appreciable difference in the rate of development of eggs and larvae in cultures maintained at salinities varying from 10‰ to 30‰ (Table II). At 5‰, however, growth was much slower, development proceeding at only half the rate at 10‰ to 30‰. Below 5‰ growth was interrupted and 2.5‰ proved to be a lethal salinity for eggs and larvae alike. The higher the temperature the more quickly the young stages died at the lower lethal salinities. The reverse was the case at the upper limit of salinity tolerance (45–50‰) where early stages survived longer at higher temperatures (for examples of similar behavior in other animals see Kinne, 1964). All growth ceased in cultures maintained at 45‰.

DISCUSSION

The data presented show clearly that the population of *S. viridis* at Lawrencetown has a short, well-defined, breeding period at the end of March and beginning of April. The observations of Dean and Pettibone of gamete-laden worms in Connecticut and New Hampshire in February, March and early April, conform to this pattern. However, the statement in the unpublished manuscript of Moore that breeding occurs in July and August at Woods Hole is conflicting. Visits to Woods Hole in July of 1963 and 1964 failed to reveal any gamete-bearing speci-

TABLE II

Effect of reduced salinity on the development of young S. viridis at 2° C. and 10° C. Data are from three experiments at each salinity. Numbers denote the setiger stage reached.

2° C.

Days after spawning	5‰	10‰	15‰	20‰	30‰
2	early cleavage	mid-cleavage	late cleavage	late cleavage	late cleavage
8	pre-trochophore	pre-trochophore	trochophore	trochophore	1
14	trochophore	1/2	2	2	2/3
20	1	3	3	3	3
26	2	3	3	3	3
32	2	3	3/4	3	3/4
38	2/3	3/4	3/4	3/4	3/4
44	3	3/4	3/4	3/4	3/4

10° C.

2	mid-cleavage	late cleavage	late cleavage	late cleavage	late cleavage
8	trochophore	1	2	1/4	2/3
14	1	2/3	3	3	3
20	2/3	3/4	4/5	4/5	4/5
26	3	6/7	7	7/8	6/7
32	3/4	8	8	8/9	8
38	4/5	9/10	10	10/11	10
44	5/6	10	10/11	10/11	11

mens of *S. viridis.* It is thus possible that Moore's observations resulted from misidentification.

The onset of gamete production which occurred during October coincided with a fall of temperature after the summer maximum in July, August and September (Fig. 23). It is well known (Orton, 1920; Gunter, 1957; Kinne, 1963) that in many animals a slowly rising or falling temperature induces gonadal development, and this may well be the stimulus for gamete production in the Lawrencetown population. According to Gunter (1957) it is usual that where temperature change is acting as a stimulus to spawning, spawning is induced by the same direction of temperature change that leads to gonadal development. In the case of *S. viridis,* however, it would appear that if temperature change is acting at all as an inducement to spawning, then it is a temperature rise in the spring that is the effecting agent. Although gamete production starts whilst temperatures are falling and continues during the coldest months of the year, it is quite possible that a rise in temperature is needed to complete the final ripening of the gametes and to bring on spawning. Indeed, if apparently mature worms are kept at a constant low temperature of approximately 0° C. they can be held for many months without spawning and will eventually reabsorb their sexual products (*cf. Crassostrea virginica*—Loosanoff and Davis, 1951). Conversely, by slowly raising the temperature from 0° C. to 2° C. over a two-week period worms can be made to spawn under laboratory conditions, up to a month before natural spawning in the field.

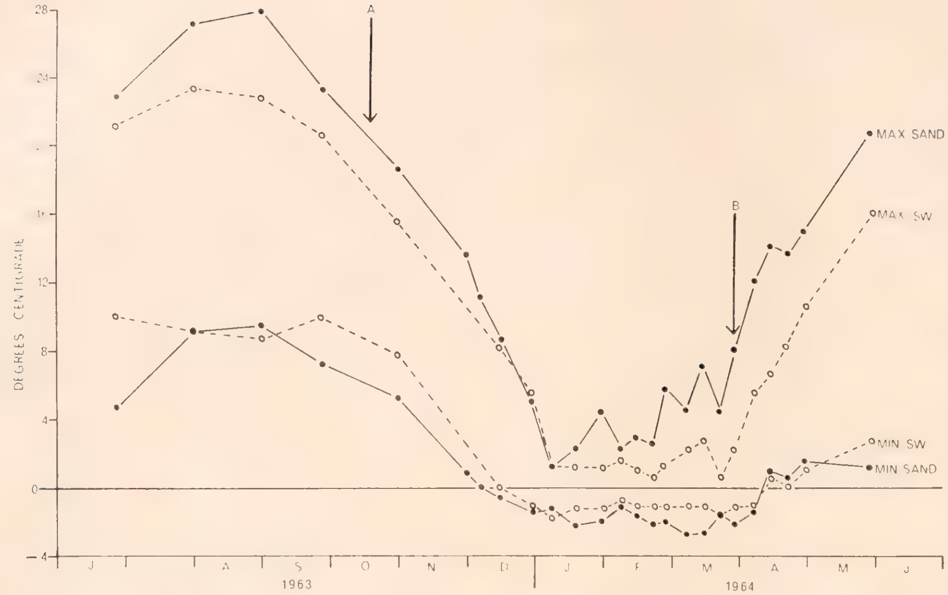

FIGURE 23. Maximum-minimum temperatures for the sea water overlying the sampling site (open circles, broken line) and for the mud-sand 1 cm. below the surface (solid circles, unbroken line). Arrows mark (A) the onset of gamete production, and (B) the time of spawning.

Sudden temperature changes seemed ineffective as a spawning stimulus (*cf. Cirriformia tentaculata*—George, 1964). On the other hand sudden changes of salinity such as occur over a tidal cycle at the sampling site induced some spawning in ripe worms.

Since spawning can be prevented by keeping the temperature at a constant low level it is unlikely that spawning occurs as a result of a biological clock mechanism or lunar periodicity.

The fact that eggs are not fertilizable nor cleavage possible below 5‰ is of interest, since 3-setiger larvae are able to live at a salinity of 2.5‰ (although not grow), and adults can tolerate a salinity of 0.5‰ for an indefinite period. A similar phenomenon has recently been reported for *Nereis diversicolor* by Smith (1964), although in these worms the range of tolerance for cleavage and successful gastrulation is narrower than for fertilization. It is likely that the susceptibility of early development stages of the worm to low salinities limits the spread of reproductively viable individuals into the upper reaches of estuaries. At Lawrencetown adults were found living permanently in areas where the salinity never rises to 5‰. These worms were presumably either passively transported there as larvae or had reached there by active swimming in the adult phase (it has already been noted that the adults are good swimmers). It is unlikely that these worms migrate back to areas of higher salinity during the breeding period, and they are probably non-reproductive individuals.

A burst of phytoplankton occurred during April of both 1963 and 1964 and continued on until the end of May. An abundance of phytoplankton at this time is extremely valuable to the larvae of *S. viridis*, ensuring them adequate nourishment when their yolk supply is exhausted. Some of the phytoplankton was of a size suitable for direct ingestion by the larvae but the increase in readily-ingestible organic detritus as a result of breakdown of the phytoplankton was probably of greater importance to the larvae.

Water temperature is also important to the Lawrencetown population, for the temperature must rise above 2° C. before the larvae have reached the 3-setiger stage or else development is suspended. Possibly this minimal temperature requirement limits the northern spread of the worm to Labrador and into the Arctic Circle, for the lower temperature tolerance levels of larvae and adults are not limiting as such. The southern limit of distribution of the worm can similarly be explained in terms of temperature. According to the surface sea-water temperature data for 1953–54 accumulated by Pyle (1962) the average August temperature in Georgia and most of the east coast of Florida exceeded 30° C., a temperature that is near the upper limit for growth of *S. viridis*. If these data are typical for these regions, then temperatures of several degrees higher can be expected as a matter of course close to shore and in estuaries. These temperatures may well exceed the upper limit of temperature tolerance (34° C.) for the species.

If spawning of *S. viridis* takes place in March and April over its whole range of distribution (as is indicated by accumulated data), then the length of time spent by the worms in the plankton will be much less towards the worms' southern limit. Using the temperature/growth curves constructed from the laboratory experiments as a guide to the variation of the growth rate with temperature, one can deduce that metamorphosis is reached in approximately four weeks in South Carolina compared with almost two months in Nova Scotia.

The author is indebted to Dr. Marian Pettibone and Dr. David Dean for supplying information and to Dr. Eve Southward for critically reading the manuscript.

SUMMARY

1. A redescription of the external morphology of *Scolecolepides viridis* (Verrill) is given.

2. From data obtained by regular sampling of a population at Lawrencetown, Nova Scotia, it was found that the worm has a short breeding period in late March and early April.

3. The onset of gamete formation coincides with a fall in the temperature following the summer maximum. Gametes occur in the coelom along the greater part of the animal's length and are shed through mixonephridia. Spawning is due to rising temperatures in the spring. In addition some spawning may result from sudden changes in salinity during a tidal cycle.

4. The thick-walled eggs containing membrane vesicles and the short-headed sperm are probably shed whilst the worms are still in their burrows. Larval development is entirely planktonic.

5. In laboratory cultures maintained at 10° C. and 30‰ photopositive trochophores begin active swimming in 3–4 days, and the 3-setiger stage is reached in 10

days. The larvae start to metamorphose at the 9- to 10-setiger stage, but metamorphosis can be postponed until the 13-setiger stage if the substratum is not suitable. The fully-metamorphosed 10-setiger worm is 750 μ long and 40 days old.

6. The rate at which development proceeds in laboratory cultures is dependent on temperature and salinity. At 20° C. metamorphosis is reached in approximately half the time taken at 10° C. At 2° C. no larvae develop beyond the 3- to 4-setiger stage. The rate of development is similar in salinities varying from 10‰ to 30‰. At 5‰, however, growth ceases.

7. An attempt is made to relate the distribution of the worm along the east coast of North America to temperature.

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FURTHER NOTES ON THE OCCURRENCE OF CHITIN IN INVERTEBRATES

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Since my previous communication on this subject (1958) I have made tests on a variety of materials, herein reported. In the meantime there has appeared the valuable book by Jeuniaux (1963) which brings together all the known facts, including an extensive tabulation of the distribution of chitin in the animal phyla. As before my tests were made by the chitosan method as given by Campbell (1929). Jeuniaux regards the chitosan test as somewhat crude and admittedly it yields only qualitative data, but is easy to carry out and requires only a few common chemicals, whereas the procedure invented by Jeuniaux must be very laborious as it requires the isolation and purification of the enzymes chitinase and chitobiase. I believe the following results obtained by the chitosan method are valid qualitatively.

Hydroids. It is generally accepted that the periderm of hydroids is chitinous. Tests made on the athecate hydroid *Tubularia* and the thecate hydroids *Obelia* and *Aglaophenia* are confirmatory; their periderm gave a good positive test.

Medusae. Hydrozoan medusa *Polyorchis* and scyphozoan medusa *Linuche* dissolved in the hot alkali and hence are devoid of chitin.

Velella. The sail of this well-known chondrophore is supported by an internal plate of cartilaginous consistency which gave a good color test.

Nematocysts. Pieces of the tentacles of *Physalia* loaded with nematocysts dissolved completely in the hot alkali, an indication that the capsular wall of the nematocysts is not chitinous as sometimes thought.

Entoprocts. Barentsia zorbunai and *Barentsia disoreta* dissolved completely in the hot alkali, hence are devoid of chitin, in sharp contrast with the ectoprocts which are mostly highly chitinous, except cyclostomes.

Cyclostomatous ectoprocts. I previously reported a negative result with the one cyclostome available, *Crisia eburnea.* I have now made tests on the following species: *Crisia eburnea, Crisia maxima, Crisina radians, Bicrisia edwardsiens, Crisulipora occidentalis, Filicrisia francanciscana, Diaperoccia californica,* and *Tubulipora tuba.* All were negative. Jeuniaux reported a feeble content of chitin in *Crisia denticulata* and attributed my negative result to the less sensitive chitosan method. In any case the chitin content of cyclotomatous estoprocts must be very small in comparison with that of other ectoprocts.

Brachiopods. Pieces of the shell of *Lingula* including the bristles gave a strong color test as previously known. An effort was made to recover the bristles, lost in the previous test; they are strongly chitinous. In previous tests on *Discinisca* the bristles also were not recovered, hence a special effort was made this time to recover them; they also gave a strong color test.

Pogonophores. Brunet and Carlisle (1958) reported the occurrence of chitin in the tubes of three species of *Siboglinum* and that of *Zenkevitchiana longissima.* However, my own test on *Polybrachia annulata* tubes, kindly presented by Professor Ivanov, was negative. Ivanov has withdrawn his statement of the presence of cellulose in the tubes of pogonophores, saying he had been misled by chemists.

Sipunculids. A test on *Golfingia gouldi* was completely negative, in agreement with Jeuniaux's finding on *Sipunculus nudus* and *Phascolosoma vulgare.*

Polychaetes. The anterior end of *Nereis virens* dissolved completely and it was impossible to find any jaws or setae in the solution. According to Jeuniaux chitin does occur in the setae of polychaetes but not in the jaws or other hard parts.

Solenogasters. The interior of a specimen of *Chaetoderma nitidulum* dissolved in the hot alkali but the body wall remained intact and gave some evidence of the presence of chitin. The radula was negative.

Chitons. Tests were made on *Chiton albolineatus, Chiton marmoratus,* and *Mopalia muscosa.* The *Chiton* species have a scaly mantle of which the scales withstood the hot alkali but gave no test for chitin. However, the mantle epidermis (cuticle?) bearing the scales is strongly positive. Similarly in *Mopalia* with spiny mantle, the spines are negative but the bristles and the mantle surface gave a strong reaction. The radula of all three species gave a strong color test. Jeuniaux reports for *Acanthochites discrepans* chitin in the bristles and the dorsal plates. I made no tests of the latter.

Gastropods. It is often stated in malacological works that the operculum of this or that snail is chitinous. This expression seems to mean that the operculum is not calcified. Tests of the decalcified operculum of *Turbo petholatus* and *Polinices duplicatus* were negative. The opercula dissolved completely in the hot alkali, and hence contain no chitin. The radulae of two terrestrial slugs, probably belonging to the Arionidae, were strongly positive to the color test. I have made no tests of the gastropod shell but according to Jeuniaux it contains chitin.

Jeuniaux regards the distribution of chitin among the animal phyla as of phylogenetic importance, basing this view on the lack of chitin in the deuterostomes and its occurrence in most of the phyla of the protostomes. He thinks the ability to synthesize chitin is an ancient property of living beings which has been secondarily lost in some animal groups.

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TRACHEAL GAS DYNAMICS IN PUPAE OF THE CECROPIA SILKWORM

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Once a Cecropia larva pupates and spins a cocoon in late summer it is destined to neither eat or drink for the rest of its life—some 8 months. It is only with the emergence of the next generation caterpillar from the egg the following spring that these activities are resumed. Strict water conservation in the pupa over this long period is obviously an urgent necessity if the insect is to avoid dehydration. The remarkable water impermeability of the insect cuticle has long been known. But the problem remains of how the insect can breathe without excessive loss of water.

The tracheal system is the pathway for oxygen and carbon dioxide exchange between the metabolizing tissues and the external environment. Because they provide such ready access for respiratory gases, the tracheal tubes are also unavoidably an easy path for water vapor loss. This, in effect, represents a shunt around the impermeable cuticle which seems calculated to dry the insect from the inside out. The insects' answer has been to open the tracheae only intermittently to the outside air; the rest of the time they are effectively sealed against the outflow of water vapor by spiracular valves (Buck, 1962).

Ideally, the spiracles should open only frequently enough to avoid anoxia to the tissues. Any additional opening represents an unwarranted water loss. Operationally, a barely sufficient ventilation is ingeniously insured by placing the spiracular valves under the control of the gases within the tracheae (Wigglesworth, 1935; Schneiderman and Williams, 1955). A combination of low pO_2 and high pCO_2 triggers the valves to open for a brief period of rapid gas exchange. The tracheae are then nearly closed off from the air for long periods.

This intermittent or "burst respiration" has received much attention (Beckel, 1958; recent review by Buck, 1962); despite this fact, much remains to be understood about the dynamics of how it works. A new analysis will be attempted here. Our chief concern will be with experimentally verifiable rather than logically reasonable hypotheses.

METHODS AND RESULTS

1. *Thermal conductivity recording*

a. CO_2 production

Study of CO_2 bursts requires that one determine the timetable for a given animal and thus be able to predict when the next burst will occur. Punt's (1944) diaferometer allowed continuous CO_2 output recording but its baseline drifted. Most recent work has been done manometrically. The laboriousness of this approach is evident when one contemplates reading a microrespirometer every 15 minutes for several days. I have developed a continuous CO_2 recorder, or diaferometer, based on the thermal conductivity changes which CO_2 produces in air. It measures the change in resistance of a heated thermistor which is placed in the gas stream.

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Oxygen and nitrogen have nearly the same thermal conductivity. That for carbon dioxide is higher, while the value for water vapor is lower. If an insect withdraws oxygen in a gas flow respirometer, there is very little change in the conductivity of the efferent gas. But if it introduces carbon dioxide or water, there can be a detectable change.

The equilibrium temperature of a thermistor with a constant electrical power input is a function of the thermal conductivity of the gas mixture surrounding it. In a high conductivity environment the thermistor can lose its heat more readily and comes to a lower temperature. With low conductivity the opposite is the case.

Two thermistors were used in a balanced bridge circuit, one being placed in the stream of gas supplied to the experimental object and the other in the efferent stream. One can readily make this arrangement record 0% to 1% CO_2 in air. It is interesting to calculate what this means in temperature difference of the thermistors. The applied electrical power heats them to about 150° C. in air. The addition of 1% CO_2 changes the temperature of one of them only 0.1°. But this is enough to provide 10 mv unbalance in the bridge and drive a recorder to full scale. With a 0.01% CO_2 sample the thermistors differ in temperature by only 0.001°. It is obvious from this that voltages and environmental temperatures of the thermistors must be carefully regulated. New solid state techniques simplify these tasks. Water vapor must also be removed after the gas stream passes the pupa and before it reaches the second thermistor.

One can automatically record every burst during weeks of metabolism. It is possible to get data from several insects at once by use of a multiple channel recorder. Animals can be quickly tested to determine if they have started developmental metabolism. Irregularities in burst period from pupa to pupa are readily apparent. Spiracular valve malfunctions show up clearly. At a chart speed of 1 inch/hour one can see the entire three-week developmental sequence of a Cecropia on a 50-foot record. Some records are presented in Figure 1.

b. Water vapor loss

The "burst mechanism" is probably of value to the insect primarily for water conservation (Beckel, 1958). Thermal conductivity can also be used to record continuously the water vapor coming from a pupa. Two thermistors are placed in the efferent air stream before and after a drying chamber in the gas stream. The change is then due only to the water vapor. With two bridges and three thermistors one can simultaneously record CO_2 and H_2O output.

Water vapor varies the thermal conductivity in the opposite direction from CO_2. The difference of its thermal conductivity from O_2 and N_2 is only half as great as CO_2. Thus the electrical signal from the bridge will be of opposite polarity and of only half the magnitude. Superimposed records of the two gases both separately and combined are shown in Figure 2.

c. Other continuous methods

An infrared analyzer also can be used to record CO_2 output. Since this technique works in the parts-per-million concentration range, it is valuable for looking at interburst CO_2 output. Such an analyzer is 2000 times more sensitive to CO_2 than to H_2O, so drying of the gas stream is not important. By recirculating the air in a closed circuit loop I was able to show the gradual increase in CO_2 concentration that results from the interburst CO_2 output of the pupa.

When the spiracles open during a burst, the rapid water vapor loss cools the pupa about 0.1° C. This cyclical cooling can be recorded by a thermistor placed on the cuticle under a tuft of cotton. The other thermistor of a bridge is also placed in the animal chamber so that ambient temperature changes produce no

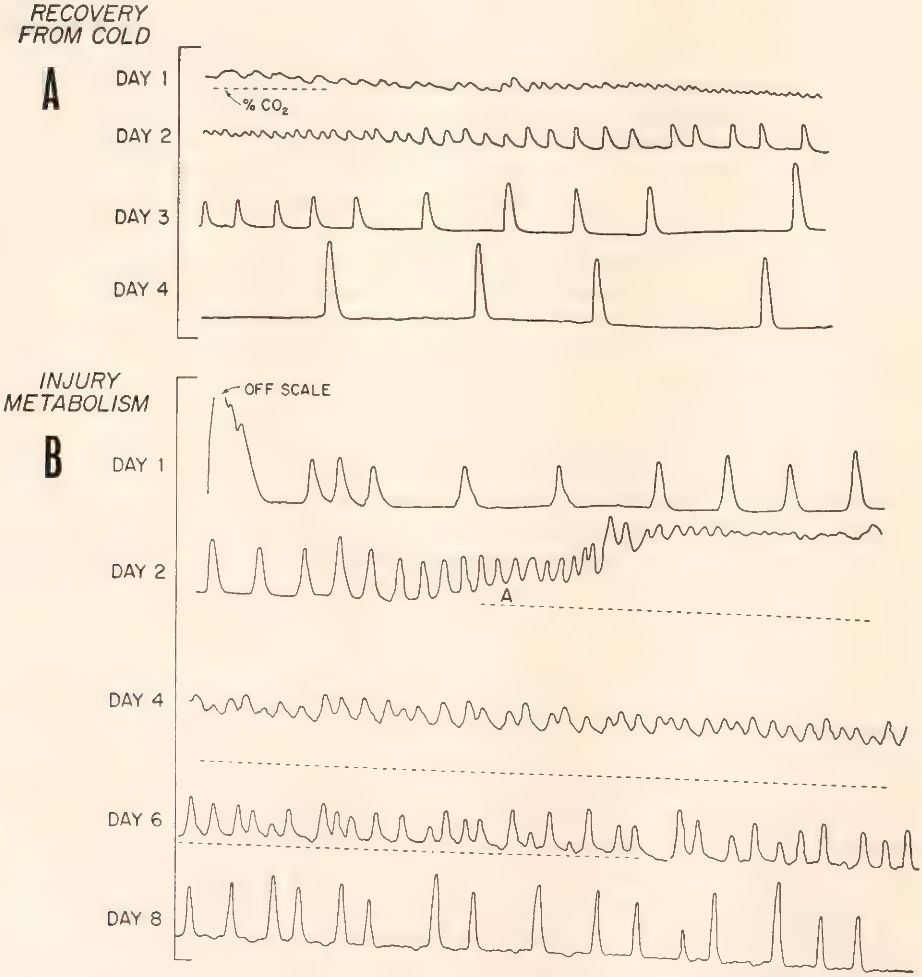

FIGURE 1. CO_2 output recording by thermal conductivity of insect pupa.

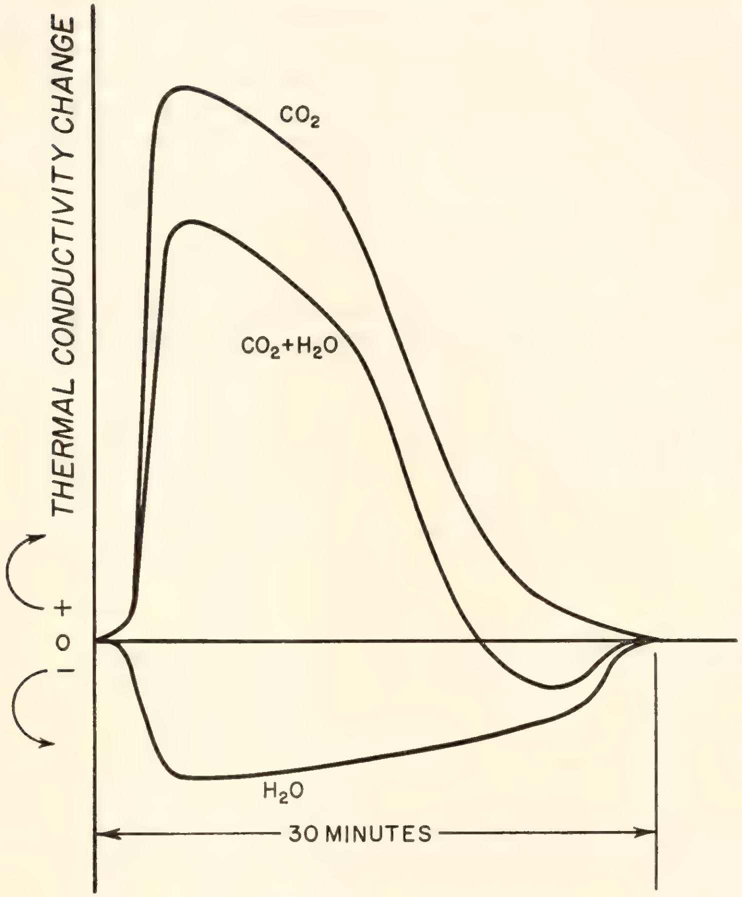

FIGURE 2. Thermal conductivity recording of CO_2, water vapor, and both combined, for a single burst.

output. This is the most simple arrangement if one only desires the time of occurrence of the bursts.

2. Tracheal-atmospheric gas exchange

A pupa consumes O_2 from the air and excretes CO_2. The CO_2 is known to come out partly in bursts. The time course of O_2 entry has not been determined directly. Manometry shows that O_2 is used internally at a constant rate. But the tracheal gas volume is a reservoir that the tissues can draw on without using outside air.

The flux of CO_2 and O_2 in and out of the spiracles has been measured simultaneously here by direct gas analysis. A pupa was suspended in a chamber of 100 to 200 ml. volume in which the air was kept at a slight positive pressure (about 3 cm. Hg). A stockcock and nipple on the top of the chamber allowed samples to be pipetted directly to a Scholander 0.5-cc. analyzer. Readings from this analyzer (Scholander, 1947) were accurate to 0.015% for CO_2 and to 0.02% for O_2. Serial readings gave the time course of the gas mixture in the animal chamber. Gradual decrease in volume as samples were withdrawn was corrected by calculation. Although this is a laborious technique, it provides definitive data on the gases passing in and out of the animal. An example of these data is shown in Figure 3.

3. *Tracheal volume and gas composition*

Tracheal volume was measured by pupal compressibility in the same way as had been applied to fish swim bladders (Kanwisher and Ebeling, 1957). The pupa was sealed in a rigid water-filled glass chamber connected to a tuberculin syringe. A trace of detergent was added to the water to eliminate surface bubbles. A small closed-end manometer in the chamber indicated pressure. The volume increments necessary to change the pressure were used to compute the volume according to Boyle's law. The pleated cuticle structure was assumed to exert no mechanical resistance to compression. The tracheal volume was about 5% of that of the entire body (average of 10 readings). For a 5-gram pupa this amounts to 250 mm.^{3}. This method gives less spread (4% to 7%) than that of Buck and Keister (1958).

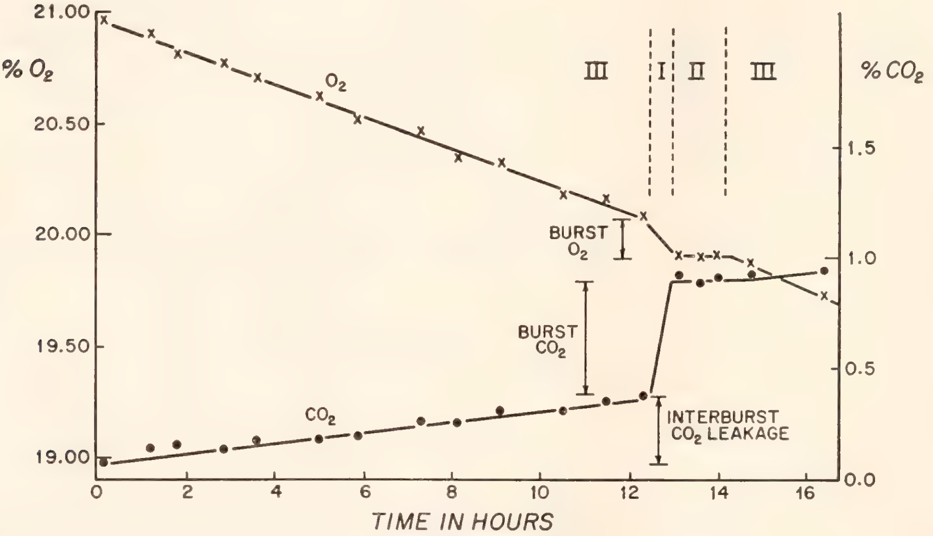

FIGURE 3. Time change of O_2 and CO_2 concentration in a sealed chamber containing a single pupa.

Levy and Schneiderman (1958) have provided the only figures on tracheal gas composition. After a ventilating burst, pO_2 and pCO_2 are, respectively, about 18% and 3%. During the initial interburst period pO_2 drops rapidly to 5% and pCO_2 increases slightly to 4%. Over the several hours until the next burst, pO_2 remains constant and pCO_2 rises to about 6%.

The tracheal gas pO_2 figures were checked by rapid vacuum extraction of the tracheal gas. A pupa in a syringe filled with concentrated citrate solution (to reduce gas solubility) was subjected to a rapid expansion. Gas visibly flowed out of some of the spiracles. This was quickly collected in an adjoining 1-ml. syringe and analyzed (Scholander *et al.,* 1955). Pupae which had just completed a burst gave off tracheal gas with 15% to 19% O_2. At the end of the interburst period the gas contained 5% to 8% O_2. The CO_2 fraction varied from 2% to 8%. Higher values were at the end of the burst period. The greater mobility of CO_2 and its buffering by body fluids make the values for this gas less reliable.

This tracheal gas analysis method required sacrificing the animal so that sequential values on a single individual were not possible. However, the values represent a fair sampling of the entire tracheal gas system since sometimes as much as 150 $mm.^3$ were obtained by this vacuum purging technique. It appears that the measurements of Levy and Schneiderman on gas samples from a cannulated spiracle are reasonably representative of the entire tracheal system. Buck (1962) surmised this homogeneity by reasoning that the main diffusive barrier is within the tissues rather than in the trachea. Buck and Keister (1958) also obtained figures for pCO_2 which are in general agreement.

DISCUSSION

1. *Spiracular gas flux*

Gas exchange between the air and the tissue takes place through the tracheal reservoir. Oxygen is in one-way transit from the air to the tissues. Warburg records show that it enters the tissues at a constant rate. Carbon dioxide moves discontinuously in the opposite direction because of bicarbonate buffering and intermittent tracheal ventilation. Nitrogen is an inert filler which passes in and out of the trachea through the spiracles to make up the volume deficit produced by CO_2 buffering and also by any departure of the R.Q. from unity. The volume of the tracheal system is assumed to remain constant.

To analyze this gas exchange system requires the coordination of the various types of data mentioned. For this purpose it is convenient to consider a burst sequence as consisting of three phases. Stage I, when the valves are open and gas exchange is rapid, lasts 15 to 20 minutes. Stage II covers roughly the first hour of the interburst period; during this time the tracheal pO_2 drops rapidly and pCO_2 increases slightly. The pN_2 increases to make up the difference. Stage III extends for the rest of the interburst period: the pO_2 remains low and constant, pCO_2 increases slightly, and pN_2 drops the same amount to make up the difference. The tracheal gas compositions (from Levy and Schneiderman, 1958) at the end of these phases are given in Table I. The changes in tracheal gas composition are also included.

From these figures we can immediately compute the flux of N_2 for a hypothetical 5-gm. pupa with a tracheal volume of 250 $mm.^3$. During the burst (stage I), 27 $mm.^3$ of N_2 flow out through the spiracles. Strictly speaking, about another 20% should be added in allowance for N_2 dissolved in the tissues. In stage II 30 $mm.^3$ flow back in. During stage III the difference of 3 $mm.^3$ must flow out.

Oxygen uptake presents more difficulties. We assume that it enters the tissues

TABLE I

Tracheal gas composition (in atmospheres)

Change during I	End of I	Change during II	End of II	Change during III	End of III
O_2 + 0.13	0.18	−0.13	0.05	0	0.05
CO_2 − 0.03	0.03	+0.01	0.04	+0.02	0.06
N_2 − 0.11	0.79	+0.12	0.91	−0.02	0.89

at a steady rate. With a reasonable diapause metabolic rate of 20 mm.^{3} O_2/gm./hr. the uptake for the whole pupa is 100 mm.^{3}/hr. During a 30-minute burst 50 mm.^{3} of O_2 must thus enter the tissues. At the same time the tracheal gas increases from 5% to 18% O_2 which requires an additional 32 mm.^{3} O_2. The sum of these, 82 mm.^{3}, must flow in through the spiracles while they are wide open for the burst (stage I).

During the hour of stage II, 100 mm.^{3} flows into the tissues, of which 32 mm.^{3} comes from change in the tracheal gas composition. The rest must come from outside. But the chamber air shows little change in composition during this time. This would be true if air rather than O_2 alone were entering the spiracles, as Buck proposes. Eventually however, the N_2 presents a disposal problem and so the pO_2 falls due to N_2 dilution. When there is only 5% O_2 left, the valves open slightly or flutter (Schneiderman, 1956). This marks the beginning of stage III during which O_2 enters by pore diffusion driven by a partial pressure difference of $0.21 - 0.05 = 0.16$ atmosphere. For the rest of the interburst period the flux of O_2 through the spiracles equals that into the tissues, and tracheal pO_2 is constant. At the next burst O_2 surges in through the open spiracles as the cycle begins again.

The changes in tracheal pCO_2 are small (3% to 6%), so the flux of CO_2 from the tissues into the tracheae must at all times be nearly equal to that out through the spiracles. Consider the case of a metabolic production of 70 mm.^{3}/hr. (R.Q. = 0.7) and a half-hour burst. During this time tracheal gas change accounts for 7 mm.^{3} flowing out. But in a pupa with a 15-hour period the CO_2 flux from the tissues during this time may be 100 times this or 700 mm.^{3}. Tracheal gas chamber buffering is clearly negligible. Most of this CO_2 outflow during such a burst must have been chemically combined in the tissues.

In stage II, negligible CO_2 is released (0.5-cc. and infrared analyzers). This is compatible with the inference from O_2 that air is flowing in during this time under a difference in total pressure. Out diffusion against this stream is apparently ineffective. A small portion of the CO_2 produced flows from the tissues into the tracheae. But bicarbonate buffering keeps most of it in solution.

If O_2 diffuses in during stage III, we should expect CO_2 to pass in the opposite direction. During this period there is an average Δ pCO_2 of 5% across the spiracular valve, or about one-third that of pO_2. Since the two gases have nearly the same diffusibility there should be one-third the flux of CO_2 that there is of O_2. This is found in both 0.5-cc. and infrared measurements. It probably matters little that the CO_2 is diffusing out against a greater counter-current of diffusing O_2.

This relatively large interburst release (350 mm.^{3} in our hypothetical case) is spread over such a long time that it is hard to see in manometric records where measurements of CO_2 and O_2 are performed in separate experiments. It also rarely shows up clearly on the thermal conductivity apparatus. This can be expected since the rate of release is only about 1% of the peak value during the burst (17 as compared to 1400 mm.^{3} CO_2/hr.). So, during this time we can say that one-third of the CO_2 produced is released to the tracheae and mostly diffuses out through the spiracles. The two-thirds remainder is buffered in the blood. This is what Buck (1958) had theorized.

In this idealized treatment the time curves for O_2 and CO_2 in a sealed chamber are shown in Figure 3. The interburst CO_2 release shows an increasing slope due to the climb from 4% to 6% in the tracheal gas. Some actual measurements are also given. They show reasonable agreement.

2. *Water vapor*

Water vapor, like CO_2, diffuses only out of the animal. We can assume that the tracheal gas is saturated. At 25° C. this means 24 mm. Hg H_2O vapor pressure, or 3% of an atmosphere. During the interburst period of stage III it will diffuse out about half as fast as CO_2 if the external air is dry. When the valves are open in stage I it will flood out like CO_2. Since the wet tissues beyond the tracheae represent a reservoir of water, the vapor pressure value of 3% in the tracheae should be maintained throughout the burst.

Thermal conductivity shows nearly this picture. The curves for CO_2, $CO_2 + H_2O$, and H_2O alone are shown in Figure 2. A given percentage of H_2O has only half the unbalancing effect on the bridge that CO_2 has. Thus, the combined curve shows a strong excursion in the CO_2 direction at the beginning of a burst.

At the end of the burst there is more H_2O than CO_2 coming out per unit time and the deflection is in the other direction. This indicates that the valves stay open slightly longer than is really necessary. Once a burst has started, pCO_2 no longer directly controls the valves. Or perhaps the triggering mechanism for closure is a slow one.

The surprising feature is that, even in a dry atmosphere, the water vapor lost is on the average about the same amount as the CO_2 produced metabolically. With fat as an energy source (indicated by an R.Q. of 0.7), this is the same as the amount of H_2O produced metabolically. The Cecropia pupa, like the desert rat, can literally produce all the water it needs. In a high relative humidity there should actually be a net gain. Since carbon is being lost, the weight should remain nearly constant. This was tested. Five pupae were kept in a jar over water for four months. They lost an average of only 5% in weight.

If anything happens to the spiracular valves in a dry environment, the insect is doomed. Water loss is now continuous at the burst rate. This means it is at least 25 times greater than before. Three pupae with the spiracular valves cannulated were exposed to 30% relative humidity. In three weeks' time they were 28% to 30% lighter, looked shriveled, and did not appear to be alive. They did not recover when subsequently kept over water.

3. Burst sequence

A large amount of data on burst time sequences has been collected. There is considerable variation in period in a group of identically treated pupae, all in diapause. At 20° C. these intervals varied from 4 to 17 hours. A given individual maintains a remarkably constant cycle for several weeks. A slight lowering of insect environmental temperature shows up as a lengthening of the burst cycle. The triggering CO_2 level of the valves remains constant so the lower metabolic rate takes longer to achieve this value in the tracheae.

When the insect is cooler than about 10°, there appear to be no bursts. Apparently pore diffusion can take care of the low gas exchange rate.

A pupa which has been at the still lower temperature of 6° shows a transiently higher metabolism for one to two days when brought to 25° C. During this time the burst mechanism is inoperative. It recovers slowly over a period of days (Fig. 1a).

In injury metabolism or during development the bursts come closer together and eventually fuse. Slight oscillations show on top of the high value of CO_2 output (Fig. 1b).

Metabolic preparations for the initiation of adult development are clearly indicated by an increase in burst frequency several days before there is any visible evidence of development. The emergence of the adult moth from the pupal skin shows up as a startling increase in CO_2 output which results from the struggle to get out of the respirometer chamber.

Conclusions

New methods have been applied to the dynamics of gas exchange in the tracheal system of pupae of the Cecropia silkworm permitting the direct observation of many features of O_2, CO_2, and N_2 flux that had previously only been deduced from indirect evidence. This system functions so efficiently that water loss is reduced to a value which is approximately the same as that produced metabolically.

This work would have been impossible without the stimulating environment provided by C. M. Williams and his students. Their generous supplying of pupae is only one of the many favors for which I am indebted.

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A COMPARATIVE STUDY ON THE CILIARY FEEDING MECHANISMS OF ANADARA SPECIES FROM DIFFERENT HABITATS

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Ciliary currents and feeding mechanisms in the mantle cavity of bivalves have been extensively studied (Kellogg, 1915; Atkins, 1936; others). One of the first to observe such ciliation in members of the Arcacea was Stenta (1903), who noted the peculiar occurrence of a posteriorly-directed rejection current along the ventral edge of the ctenidia in *Arca*. Atkins (1936) examined in detail the currents associated with the ctenidia, palps, visceral mass, and mantle of *Glycymeris glycymeris* (L.) and *Arca tetragona* Poli and reported the presence of latero-frontal cilia in both these species. About 20 years later Yonge (1955) found that the inhalant and exhalant currents were restricted to the posterior region in "*Arca senilis* Lam." (= *Anadara senilis* L.). Purchon (1956) has briefly described the ctenidia, palp, and mantle ciliation in *Anadara granosa* (L.), and reported an anteriorly-directed ciliary current along the ventral ctenidial edge instead of a posteriorly-directed rejection current which is apparently universal within the Arcacea. Recently the general pattern of the ciliary currents has been examined in *Anadara trapezia* (Deshayes) by Sullivan (1961), who confirmed the findings of Atkins (1936).

In this study the ciliary feeding mechanisms of three species of *Anadara* from three different habitats (namely, rocky, sandy, and muddy) were investigated to find out if there were any significant differences. Observations were also made on *A. granosa* previously studied by Purchon (1956).

MATERIALS AND METHODS

Specimens of *Anadara antiquata* (L.), *A. anomala* (Reeve), and *A. cuneata* (Reeve) were collected from rocks, sand, and mangrove mud in Singapore at Tanjong Gul, Bedok, and Seletar, respectively. They were all obtained from the intertidal zone of the shore and examined in the Department of Zoology, University of Singapore. Specimens of *A. granosa* were obtained from Singapore markets.

Living specimens in sea water were treated with particles of animal charcoal, carborundum and carmine, and the movements of these particles were studied under the microscope. In all cases the right valve, including the right mantle lobe, was first carefully removed before observations were made within the mantle cavity. After these *in situ* studies, portions of the ctenidia and labial palps were excised and examined separately under the microscope in the same manner.

The inhalant and exhalant currents through the mantle cavity of intact living animals were investigated in small glass tanks. Carmine particles were employed and the conditions in the tanks were made to resemble those in the field as far as possible.

RESULTS

FIELD OBSERVATIONS

The three species, *Anadara antiquata, A. anomala,* and *A. cuneata,* were observed to exhibit vertical zonation, although occasional individuals may be found in any part of the shore that is inundated. *A. cuneata* occurs between mean sea-level and high water of spring tides. *A. antiquata* usually occurs around mean sea-level. *A. anomala* is restricted to areas below mean sea-level.

Anadara antiquata, being a rocky-shore inhabitant, is usually found attached to rocks and stones by means of its slender byssus. Its position is variable, depending upon the contour of the rock or stone to which it is anchored. In any case the posterior portions of the animal are nearly always more or less oriented towards light (Fig. 1A). The byssus is detached very easily if not carefully handled. The

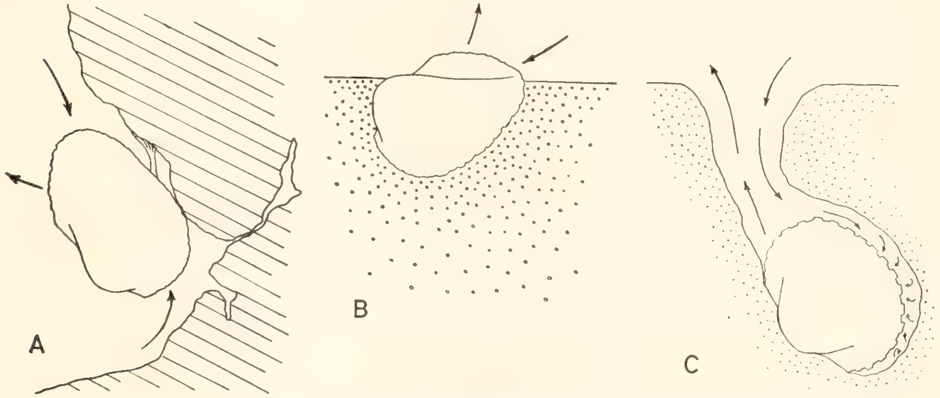

FIGURE 1. Water currents entering and leaving the mantle cavity (position in life). A. *Anadara antiquata* (L.). B. *Anadara anomala* (Rve.). C. *Anadara cuneata* (Rve.).

ABBREVIATIONS USED FOR FIGURES 1 TO 5

A, anus; Al, amoebocyte; AA, anterior adductor muscle; AID, ascending limb of inner demibranch; AOD, ascending limb of outer demibranch; ARM, anterior retractor muscle; B, byssus; BG, byssal groove; BS, blood sinus; CFC, coarse frontal cilia; CJ, ciliary junction; CT, connective tissue; DID, descending limb of inner demibranch; DOD, descending limb of outer demibranch; E, "eye" spots on edge of mantle lobe; F, foot; FFC, fine frontal cilia; GA, gill axis; IE, inner epithelium; IF, inner fold of labial palp; IS, infra-filamental septum; LID, left inner demibranch; LC, lateral cilia; LM, left mantle lobe; LOG, lateral oral groove; M, mouth; MF, membranous fold of labial palp; MF1, muscle fiber; MG, mucous gland cell; MG1, mucous gland cell of first type; MG2, mucous gland cell of second type; MG3, mucous gland cell of third type; NE, nucleated erythrocyte; OE, outer epithelium; OFC, orally directed food current; OP, outer fold of labial palp; PA, posterior adductor muscle; POG, proximal oral groove; PRC, posteriorly directed rejection current; PRM, posterior retractor muscle; RIP, inner fold of right labial palp; ROD, right outer demibranch; ROP, outer fold of right labial palp; RP, portion of right labial palp; SM, suspensory membrane; TF, transverse fold of labial palp; TG, transverse groove of labial palp; VM, visceral mass; X, point of rejection of particles; Y, point of entry of current from outer surface of palp.

point of detachment is close to its origin within the byssal groove. When the animal is dislodged, it moves with the help of its muscular foot and re-attaches itself with the secretion of a new byssus. Frequently, a commensal crab (*Pinnotheres* sp.) is seen living in the mantle cavity of the bivalve.

Unlike *A. antiquata* the species *A. anomala* lies buried with the greater part of its body embedded in the sand, with only a little of the posterior end protruding above the surface (Fig. 1B). Growths of algae and tiny barnacles often encrust the exposed posterior end of the shell. The byssus is absent. Although the former species (*A. antiquata*) may be wholly exposed to air at low tide, *A. anomala* never experiences complete exposure. It remains in its semi-buried position most of the time and is not observed to change its burrow unless dislodged. *A. anomala* is usually found on sandy shores, where the closely related burrowing species, *A. inaequivalvis* (Bruguiere), also occurs.

The large species *A. cuneata* lives in the estuarine mangrove mud of Seletar and some parts of the Johore Straits. Unlike the two previous species, it lies completely buried in mud. It is a shallow burrower and is found 6–10 cm. below the mud surface. The entrance to the burrow is irregular in shape and measures roughly 2–3 cm. in diameter In its shallow burrow, the bivalve lies with its ventro-posterior facing uppermost (Fig. 1C).

Currents entering and leaving the mantle cavity

A. *Anadara antiquata*

The currents leaving and entering the mantle cavity resemble very closely those of *Glycymeris glycymeris* and *Arca tetragona* as observed by Atkins (1936). There are two major inhalant currents and one exhalant current (Fig. 1A). The inhalant currents appear at the anterior and ventro-posterior regions and the exhalant one is observed at the dorso-posterior region of the valves.

B. *Anadara anomala*

There are a single inhalant current (as contrasted to two in *A. antiquata*) and one exhalant current. Both the inhalant and exhalant currents lie posteriorly (Fig. 1B) as in *A. senilis,* which was studied by Yonge (1955).

C. *Anadara cuneata*

The results of observations on this species agree with those obtained by Sullivan (1961) for *A. trapezia.* There is present a distinct exhalant current at the dorso-posterior region while the inhalant current appears to enter the mantle cavity diffusely throughout the ventral margin of the shell. However, the major part of the inhalant water appears to enter the anterior region of the ventral gape (Fig. 1C).

The ctenidia and their ciliary currents

Structure

The anatomy and histology of the ctenidia of *A. antiquata, A. anomala* and *A. cuneata* show the general characters of the filibranch gill as observed by Ridewood (1903). In all three species studied, latero-frontal and aboral cilia are absent. There are no significant differences in the anatomy and histology of the ctenidia among each of the three species in this study and *A. trapezia* as described by Sullivan (1961).

Some slight anatomical and histological differences do occur among the three species investigated. Comparatively the size of the ctenidia is largest in *A. cuneata* —volume for volume of mantle cavity. There is no appreciable difference in the size of the ctenidia in *A. antiquata* and *A. anomala* (see Figs. 3E, 4E and 5E). The position, shape and flexure of the demibranchs are very similar in each of the three species. The fine frontal cilia are fewer in number in *A. antiquata* than in *A. cuneata,* with *A. anomala* having an intermediate number. Consequently, there is a proportionate migration of the lateral cilia from a more peripheral region in *A. antiquata* to a more central position in *A. cuneata.* The lateral cilia in *A. anomala* show more or less an intermediate condition between the other two species (Figs. 3A, 4A, and 5A).

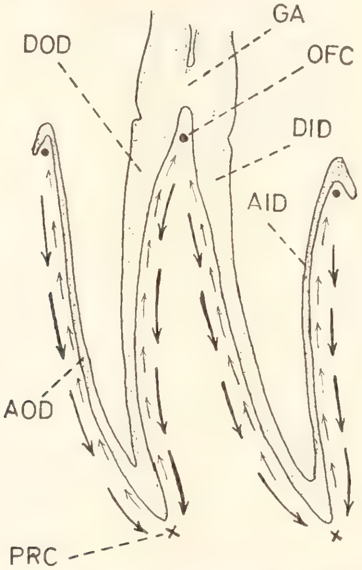

FIGURE 2. Semi-diagrammatic transverse section of ctenidium, showing the direction of the ciliary currents in *Anadara.*

The ciliary currents

Stenta (1903) and later Atkins (1936) found that, unlike the usual anteriorly directed current along the ventral margin of the ctenidia, the marginal current in *Arca* was directed posteriorly. The tract with posteriorly-directed ciliary currents was also noted by Yonge (1955) and Sullivan (1961) for *Anadara senilis* and *A. trapezia,* respectively. The three species of *Anadara* here studied have also been found to show this peculiarity (Figs. 2, 3E, 4E, and 5E). The dorsal, orally

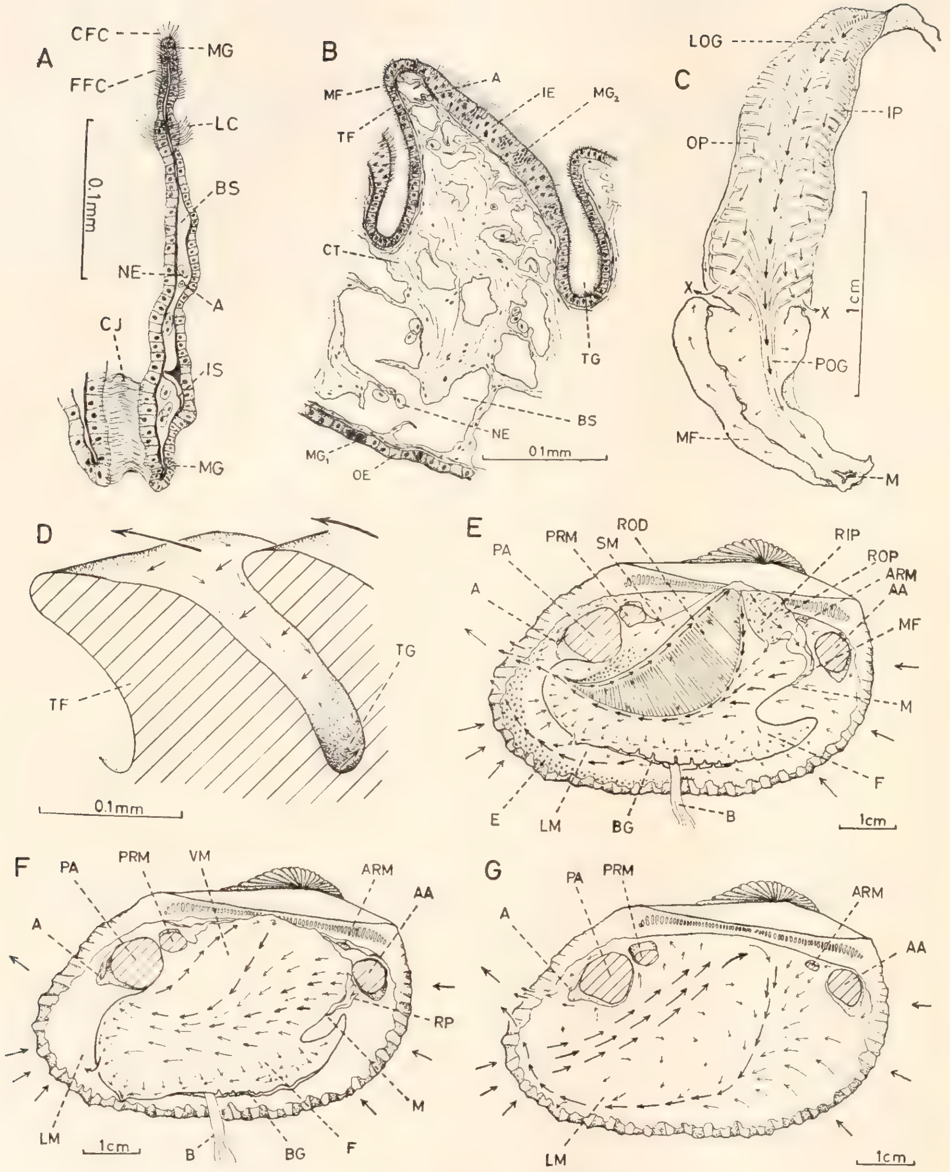
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FIGURE 3. *Anadara antiquata* (L.). A. Transverse section of gill filament. B. Transverse section through transverse fold of labial palp. C. Ciliary currents of the left labial palp. D. Currents on two adjacent folds of labial palp (diagrammatic). E. Currents on ctenidium and adjacent areas. F. Currents on the foot and visceral mass. G. Currents on the left mantle lobe.

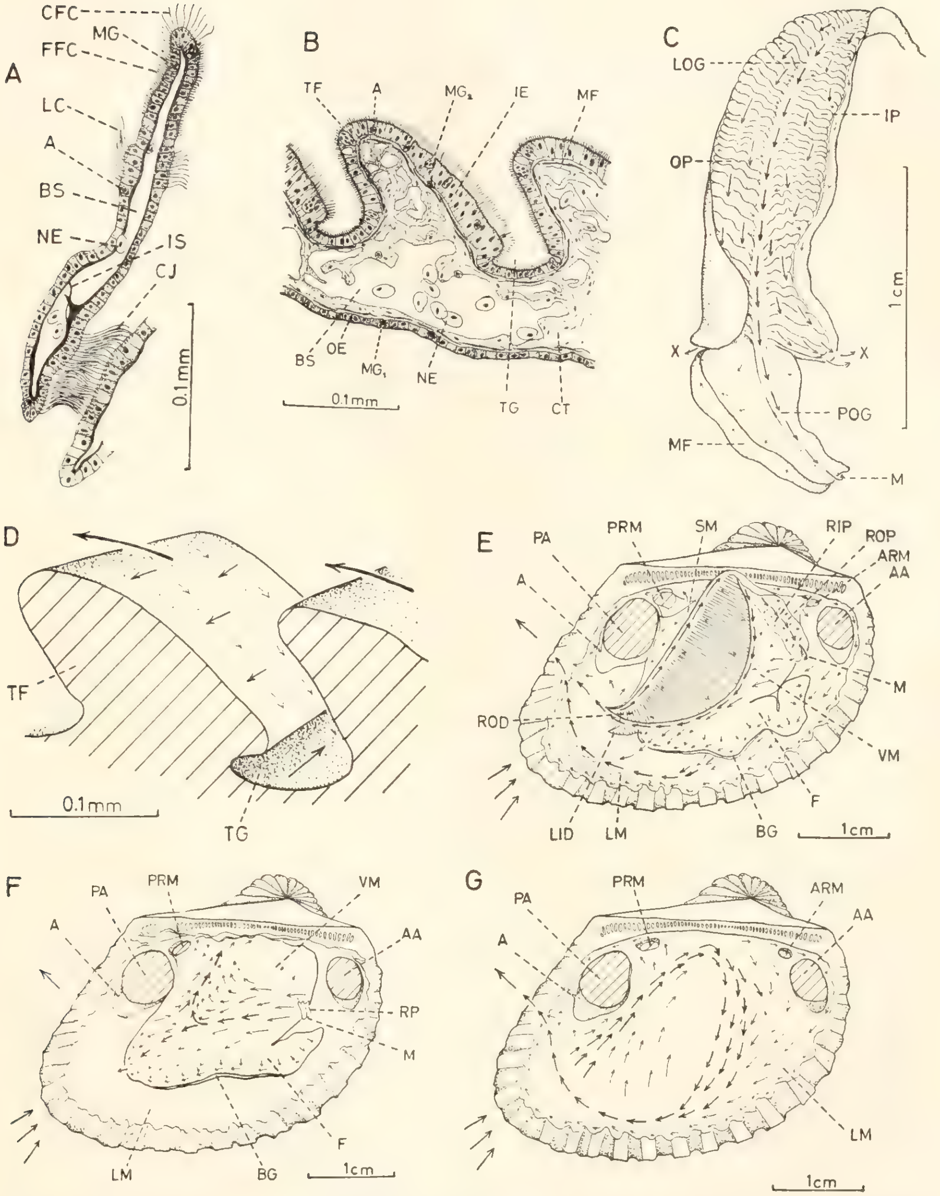
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FIGURE 4. *Anadara anomala* (Rve.). A. Transverse section of gill filament. B. Transverse section through transverse fold of labial palp. C. Ciliary currents of the left labial palp. D. Currents on two adjacent folds of labial palp (diagrammatic). E. Currents on ctenidium and adjacent areas. F. Currents on the foot and visceral mass. G. Currents on the left mantle lobe.

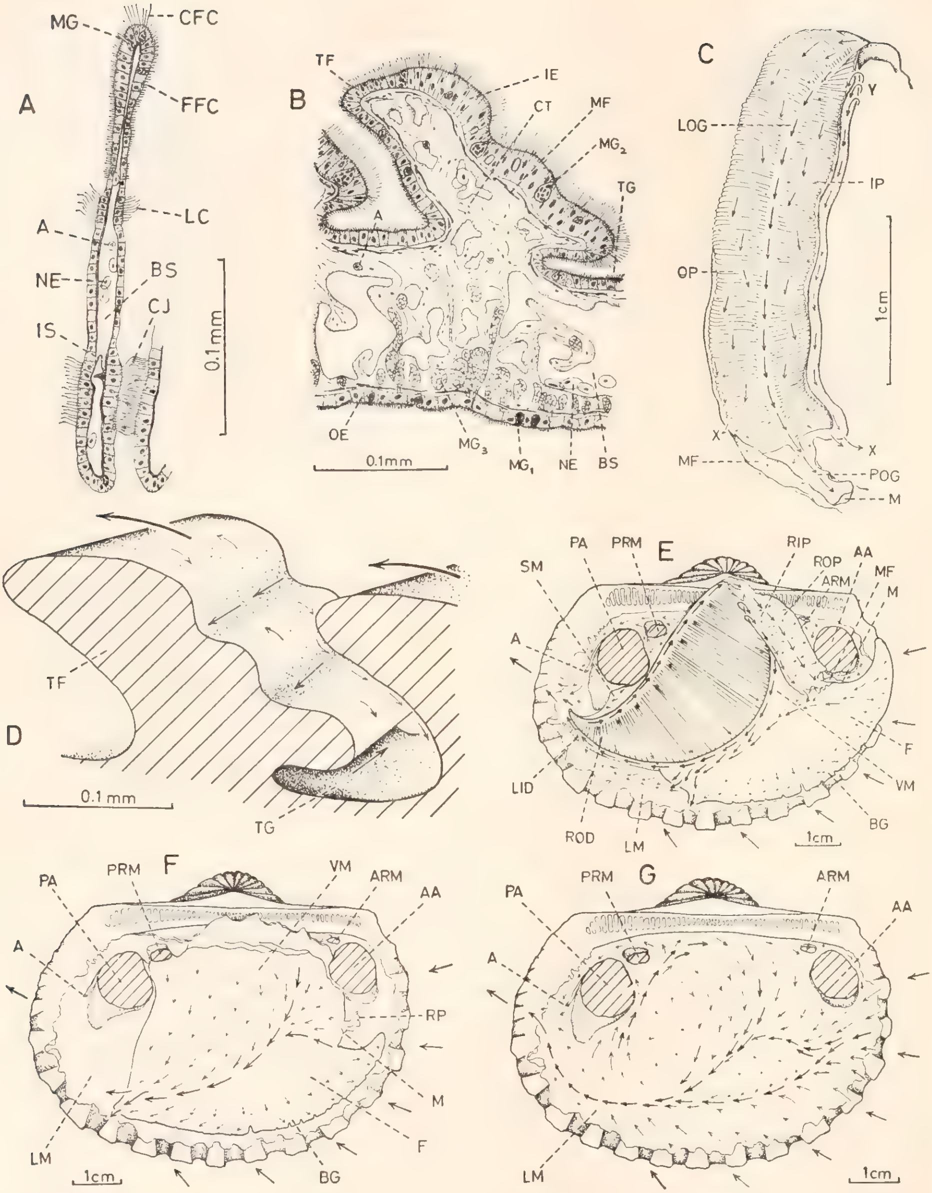
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FIGURE 5. *Anadara cuneata* (Rve.). A. Transverse section of gill filament. B. Transverse section through transverse fold of labial palp. C. Ciliary currents of the left labial palp. D. Currents on two adjacent folds of labial palp (diagrammatic). E. Currents on ctenidium and adjacent areas. F. Currents on the foot and visceral mass. G. Currents on the left mantle lobe.

directed, food (ciliary) current and the sorting (ciliary) currents of the gill lamellae of *A. antiquata, A. anomala* and *A. cuneata* were all similar to those observed by Atkins (1936) in *Glycymeris* and *Arca.*

My own observations of the ciliary currents in *A. granosa* show that the ciliary current on the ventral margin of the ctenidia in this species is the reverse of what was reported by Purchon (1956) ; his "weak (food) ciliary current" (Purchon 1956, p. 64) is in fact a strong posteriorly directed rejection current.

The labial palps and their sorting mechanisms

A. *Structure*

The labial palps show more differences among *A. antiquata, A. anomala* and *A. cuneata* than the gills. The ventral tips of the anterior filaments of the inner demibranchs of all the three species examined are not inserted into the distal oral groove of the labial palp. This ctenidium-palp association would be classified under Category III of Stasek (1963).

There are significant interspecific differences in the number of the three species examined. *A. antiquata* has 40, *A. anomala* has 43, and *A. cuneata* about 150 transverse folds. These may be compared with 1–3 folds in *Glycymeris glycymeris,* about 12 folds in *Arca tetragona,* and about 36 folds in *Anadara trapezia* (Atkins, 1936; Sullivan, 1961). The transverse folds are slender and long in *A. cuneata* (Fig. 5C) but thicker and broader in the other two species. The folds of *A. antiquata* (Fig. 3A) are relatively simple and straight, while those of *A. anomala* are wavy (Fig. 4C). (I have observed a similar type of wavy palps in *A. inaequivalvis* (unpublished).) Furthermore, the individual folds also show specific differences. For instance, while the folds remain simple in *A. antiquata,* a ridge occurs at the region of the transverse groove in *A. anomala* and *A. cuneata* (Figs. 3D, 4D and 5D). Moreover, the transverse-fold surface is doubly-grooved in *A. cuneata.* The labial palps of *A. granosa* (Purchon, 1956; and my own observations) reach a stage of complexity intermediate between *A. anomala* and *A. cuneata.*

B. *Histology*

The inner epithelia of all the three species are ciliated, columnar, and variable in height. The epithelia of the outer surface are all cuboidal and are only finely ciliated in *A. cuneata* (Figs. 3B, 4B and 5B). No such inner surface ciliation was observed in *A. trapezia* (Sullivan, 1961). Mucous glands are present in the epithelia of the labial palps of all the species examined. They are unicellular and multicellular. The unicellular mucous glands are of two types, MG1 and MG2. The cytoplasm of MG1 contains few small, indistinct, vacuoles. The cytoplasm of MG2 is very distinctly vacuolated. The multicellular mucous glands or MG3 have vacuolated foamy cytoplasm. The MG1 and MG2 cells are observed in both *A. antiquata* and *A. anomala* (Figs. 3B and 4B) and MG1, MG2 and MG3 cells in *A. cuneata* (Fig. 5B). The MG1, MG2 and MG3 cells are similar in structure to the PA, PB and PC cells of *A. trapezia* as described by Sullivan (1961). In *A. trapezia,* the PC (= MG3) cells occur in the smooth proximal portion of the inner surface, while in *A. cuneata* the MG3 cells occur throughout the outer surface of the labial palp (Fig. 5B).

The sorting mechanisms

The sorting mechanisms on the inner surface of the labial palps are similar to those observed by Atkins (1936) for *Arca* and *Glycymeris,* by Sullivan (1961) for *Anadara trapezia,* and Purchon (1956) for *A. granosa.* They all receive particles of food, etc. from the ctenidia and further sortings are conducted on the inner surfaces of the palps (Figs. 3C, 4C and 5C) prior to any entry (of the particles) into the mouth. The main currents (very large arrows in Figs. 3D, 4D and 5D) carrying particles from the ctenidia pass down the upper surface of the palps and leave at the point "X" in Figures 3C, 4C and 5C. As the main currents proceed along, subsidiary ciliary currents of the transverse fold surfaces (large and small arrows on transverse folds in Figs. 3D, 4D and 5D) sort out the rejected particles, passing them on to the current on the outer-edge of the palp. The accepted particles are conveyed to the transverse groove. The outer-edge current collects the rejected particles from all the transverse folds and ferries them to "X." The transverse groove currents (strong arrows in transverse grooves in Figs. 3D, 4D and 5D) join the lateral oral groove current into which they pass the accepted particles. The non-folded surfaces of the proximal portions of the palps have small, rather diffuse, ciliary currents, as shown in Figures 3C, 4C and 5C, where particles are mainly for rejection and are directed towards the position of "X." As in *A. granosa* (Purchon, 1956) there is a strong ciliary current carrying particles collected from the outer surface of the labial palps onto the inner surface at the distal portion in *A. cuneata* (point "Y" in Fig. 5C). This is absent in *A. antiquata* and *A. anomala,* and was not observed by Atkins (1936) and Sullivan (1961) for the species they studied.

Ciliary currents on the foot and visceral mass

After removal of the ctenidium and labial palp on one side, the ciliary tracts on the visceral mass and foot were observed. There are dissimilarities among all the three species investigated. The arrangement in *A. antiquata* appears to be somewhat similar to that seen in *Arca tetragona* (Atkins, 1936), where there are discernible dorso-anterior and ventro-posterior tracts. These roughly coincide in position with the two major ctenidial tracts (Fig. 3F). The situation is somewhat modified in *A. anomala.* In this species are a dorsally-directed current, roughly along the position of the mid-portion of the gill lamellae, and a ventro-posterior current (Fig. 4F). A distinct dorso-anterior current is absent in *A. cuneata* (Fig. 5F). The posteriorly directed rejection tract leaves the foot and visceral mass in roughly the same position in all the three species as in *Arca* (Atkins, 1936).

Ciliary currents on the mantle lobe

Ciliary currents on the mantle were observed after careful removal of the ctenidia, labial palps, foot and visceral mass.

Basically, the ciliary tracts on the mantle lobe in all the three species examined are similar (Figs. 3G, 4G and 5G). There are present distinct dorso-anterior and ventro-posterior currents not unlike those of *Arca* (Atkins, 1936) and *Anadara trapezia* (Sullivan, 1961). These two currents roughly correspond with the ones of the ctenidium in position. In *A. cuneata* the ventro-posterior tract is reinforced by a third distinct tract—the mid-posterior. This current gathers the dorso-anterior current ventrally and enters into the ventro-posterior current more posteriorly. In *A. granosa,* although there is no dorso-anterior current on the mantle, a diffuse dorso-posterior flow and a ventro-posterior current occur (Purchon, 1956; my own observations).

The position of rejection from the mantle lobe and subsequent expulsion from the mantle cavity to the exterior lies posteriorly (see Figs. 3G, 4G and 5G) in all the species studied. This more or less coincides with the region of exit of the exhalant current. In *Arca* the point of expulsion happens to be in the region of the posterior inhalant current where particles are consolidated (Atkins, 1936) before ejection through the inhalant opening by "clapping" of the shell valves.

Discussion

The investigations in the field and the laboratory on the ciliary feeding mechanisms indicate very strongly that the species *Anadara antiquata, A. anomala* and *A. cuneata* are well suited to their respective habitats—rocky, sandy, and muddy. The inhalant and exhalant currents leaving and entering the animals show signs of adaptation to the habitats. The rocky-shore form (*A. antiquata*) is the least specialized of the three. Its form, presence of a byssus throughout life, and its three distinct inhalant and exhalant currents (one anterior and two posterior) closely resemble those of *Arca* (Atkins, 1936), which is a more primitive genus than *Anadara.* Yonge (1962) has hypothesized that byssal attachment is primarily a characteristic adaptation of metamorphosing spat, and its retention in some adult bivalves is neotenic.

The sandy-shore form (*A. anomala*) and muddy estuarine form (*A. cuneata*) are more or less specialized, from the evidence of their ciliary feeding mechanisms and associated structures. The "mud" form burrows completely and successfully retains a rather diffuse anterior inhalant current of water which brings in finely stirred-up organic particles in the mud that probably serve as the major food source instead of the plankton. The "sand" form buries itself almost completely, but not quite. A little of the posterior portion of the bivalve is left exposed. This is a plankton feeder and an anterior inhalant stream is impractical and hence absent. Yonge (1955) asserted that (p. 207) "in the Lamellibranchia the inhalant aperture is primitively anterior" when he described the total absence of an anterior inhalant current in *A. senilis.* In this respect, the species *A. anomala* is more "advanced" than *A. cuneata* since the latter still maintains traces of anterior inhalant currents.

Although the species *A. cuneata* is more "primitive" than *A. anomala,* on the basis of position of the inhalant apertures, the former shows more elaborate structures associated with feeding. The ctenidia are comparatively larger in surface area. In *A. cuneata* the labial palps have more than four times the number of transverse folds, and the individual folds are more elaborately grooved and more abundant in variety and quantity of mucous glands than in *A. anomala.* Cilia are present on the outer surface of the labial palps in *A. cuneata* but absent in *A. anomala.* The fine frontal cilia are more numerous in each gill filament in the former species than the latter. Finally, the ciliary currents on the mantle lobe of *A. cuneata* are more elaborate and there is present a mid-posterior current which is absent in *A. anomala.* All these elaborations of the feeding apparatus in the "mud" species are presumably necessary for the survival in its particular habitat. To cope with an environment of much silt and suspended particles in the surrounding water *A. cuneata* requires a bigger sorting area on the gills, labial palps, more mucous secretion and ciliation of exposed parts. Moreover, in *A. granosa* (Purchon, 1956; my own observations) and in *A. cuneata,* there are additional strong ciliary currents ("Y" in Fig. 5C) on the labial palps.

An interesting feature of ciliary currents on the gills of all members of the Arcacea studied is the presence without exception of a posteriorly directed rejection current on the ventral margin of the gills (Atkins, 1936, on *Arca tetragona* and *Glycymeris glycymeris;* Yonge, 1955, on *Anadara senilis*; Sullivan, 1961, on *A. trapezia;* and my observations on *A. antiquata, A. anomala,* and *A. cuneata*). My re-examination of *A. granosa* indicates the presence of this feature, contrary to the negative findings of Purchon (1956). Presumably this is a universal character of the family Arcidae, and possibly of the superfamily Arcacea. Except for the Anomiacea (Atkins, 1936; Yeo, personal communication) the presence of the ventro-posterior rejection current has not been found elsewhere in the Bivalvia. The separation of the Subclass Protobranchia (Order Nuculacea) from the Subclass Lamellibranchia (including Arcacea and many other more typical lamellibranchiate bivalves) is firmly based on paleontological and functional grounds (see Cox, 1959; Yonge, 1959). However, the taxodont dentition of the arcids probably has phylogenetic significance *within* the Subclass Lamellibranchia. On the other hand, the similarities of rejection currents in the anomiids and the arcids are probably totally independent in evolution, and of functional, rather than *phyletic,* significance.

SUMMARY

1. The ciliary feeding mechanisms of a rocky- (*A. antiquata*), sandy- (*A. anomala*), and muddy- (*A. cuneata*) habitat species of *Anadara* are observed.

2. Adaptive differences are found for the three species in their inhalant/exhalant apertures, structures and ciliary surfaces associated with feeding.

3. Some primitive and specialized features of the three species in relation to their feeding mechanisms are discussed.

4. Observations of the ciliary feeding currents were also made on *Anadara granosa.*

5. The ciliary tract on the ventral margin of the ctenidium is directed posteriorly in *A. granosa,* contrary to previous observation.

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TEMPERATURE EFFECTS IN REPRODUCTION OF THE BAY SCALLOP, AEQUIPECTEN IRRADIANS LAMARCK[1]

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The environmental temperature has been considered to be the most important factor in regulating the breeding period in marine invertebrates (Orton, 1920; Runnström, 1927). Korringa (1957) directed attention to variation in temperature requirements of populations of a species from different geographic localities. There is considerable discussion as to how the temperature controls the reproductive activities of marine invertebrates, and recent reviews by Giese (1959) and Boolootian (1964) include relevant literature on this subject. Breeding condition was induced in the oyster, *Crassostrea virginica* Gmelin, during winter by gradually raising water temperature and providing food (Loosanoff and Davis, 1952). The oocytes of the scallop, *Pecten yessoensis,* were accelerated to maturity *in vitro,* by exposure to higher temperatures, long before the population in nature reached maturity (Yamamoto, 1951). Arctic barnacles, *Balanus balanoides* L. and *Balanus balanus* (L.) were reported to require maintenance at 3°–10° C. for one to three months before they could be brought to a breeding condition, while the population in nature was not breeding (Crisp, 1957). Tropical species of barnacles were brought to maturity in winter by maintaining them at higher temperatures and providing food (Patel and Crisp, 1960). Maturation and spawning was advanced in the bay scallop, *Aequipecten irradians* Lamarck, by exposing the animals to higher temperature during winter, while spawning in the natural population normally begins in August (Sastry, 1963). Although these studies indicate that temperature affects the reproductive activities of marine invertebrates, it is not clear to what extent it influences the events in the reproductive cycle or its exact role in determining the annual reproductive pattern. The present study was undertaken to determine the effect of temperature on the events in a reproductive period of the bay scallop, *A. irradians* Lamarck.

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MATERIALS AND METHODS

Throughout this study, the bay scallops, *A. irradians* Lamarck, were collected from the same grass flat in the vicinity of the Duke University Marine Laboratory,

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Beaufort, North Carolina. The local population has an annual reproductive cycle and individuals commence spawning at the age of one year (Gutsell, 1930). The life span of the species is about two years throughout its range of distribution on the Atlantic and Gulf Coasts of the United States (Belding, 1910; Gutsell, 1930; Sastry, 1961). The animals in the second year are not found in large numbers, either because of commercial fishing at the end of the spawning period or due to natural mortality. Therefore, all the animals used in this study belonged to a year class and were collected from the beginning of their first reproductive period.

Analysis of field animals

After each collection, the animals were brought into the laboratory and gonad index (gonad weight/body weight × 100) and digestive gland index (digestive gland weight/body weight × 100) were determined for a sample of ten animals (Giese, 1959). The digestive gland and the gonad were easily separated from the rest of the body. The bay scallops are functional hermaphrodites, and testis and ovary occupy the proximal and distal regions of the same gonad mass. The gonad condition of each animal in the sample was assayed by microscopical examination of freshly made smears of testicular and ovarian tissues. The oocytes in the ovary were measured with an ocular micrometer in several microscopical fields, and the stage of their development was noted. The gonads were preserved for future histological study.

Laboratory procedure

In some collections, the remaining animals were divided into groups of equal size and maintained at various temperatures covering the annual temperature range characteristic of this region. The gonad coloration of each animal was examined before introducing them to the experimental temperatures. The animals were placed in battery jars containing 7 liters of sea water and maintained at different temperatures. The sea water in the containers was continuously aerated. Initially, the temperature of sea water in the battery jars was allowed to reach the desired experimental temperature over a period of time. On the alternate days, the animals were changed to fresh sea water of equilibrated temperatures. The photoperiod was regulated to provide 12 hours of light and 12 hours of darkness. The salinity of sea water varied between 25‰ and 35‰ during these experiments. No food was supplied to the animals during the entire period of this study.

Temperature effects were assessed by using the following criteria: spawning of animals at the temperature at which they were maintained; changes in gonad coloration; 50% survival of experimental animals at the respective maintenance temperatures was considered as completion of the experiment. At the end of the experiment, the remaining animals were removed and the gonad and digestive gland indices and the microscopical condition of the gonad were determined as described for the field sample. When gametes were released during the experimental period, they were fertilized and development to the veliger stage was followed. Gonads removed from the experimental animals were also preserved for future histological study. The same procedure was followed whenever the animals were maintained at different temperatures in the laboratory from the collections made at intervals during the reproductive period.

Observations on Field Animals

Changes in gonad and digestive gland indices

The mean gonad and digestive gland indices obtained for the sample analyzed at each collection during the reproductive period are shown in Figure 1. The gonad index rapidly increased during May to early July by about three times. The gonad index remained about the same from early July to early September. Towards the later part of September, the gonad index was about four times greater than in May. The gonads of marine invertebrates increase in weight by accumulation of various biochemical reserves when they reach a mature condition (Giese, 1959). A decreasing trend followed the maximum observed in the later part of September and reached a lower value by late November. The spawning period covered approximately two months during September and November. The slight variation observed in the gonad index values in each sample during gonad growth suggests that some individuals might be slower in gathering food or accumulating reserves in the gonads. A similar variation in the gonad index values during spawning period indicates that all the animals in a population might not liberate gametes at the same time, or that they liberate different amounts at any single discharge (Table I).

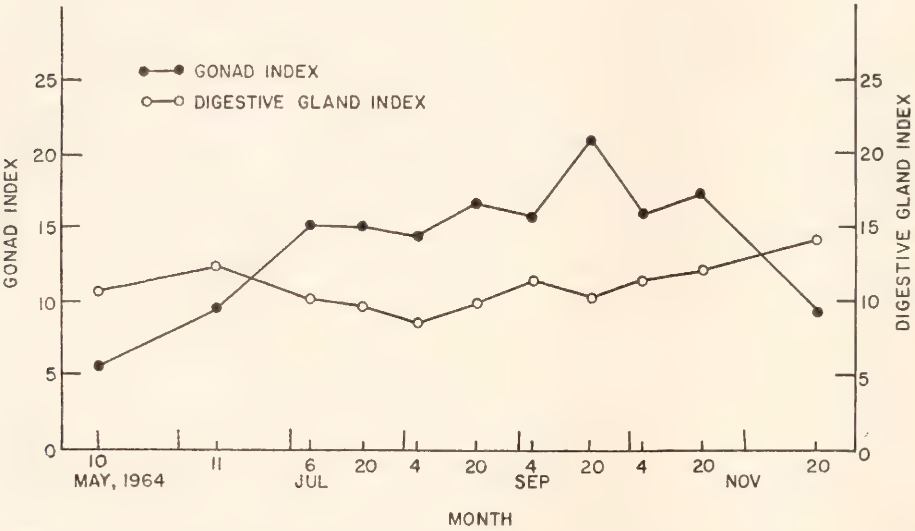

Figure 1. Changes in mean gonad index and digestive gland index of scallops during reproductive period. Note the reciprocal relationship between gonad index and the digestive gland index.

The digestive gland index was higher than the gonad index in May and early June. The digestive gland index decreased to a value less than gonad index by

Table I

Changes in gonad index, digestive gland index and the microscopical condition of gonads in A. irradians during the reproductive period

Date collected	Size mm. mean ±S.D.	Average weight g.	Average body weight g.	Gonad index mean ±S.D.	Digestive gland index mean ±S.D.	Gonad condition		
						Average oocyte diameter μ	Color	Microscopical
5/10/64	33.84 ±4.49	11.33	3.39	5.5 ±1.1	10.75 ±1.72	8.0; range 5.2–13.0	dark	spermatocytes, oogonia, few early oocytes
6/11/64	39.69 ±3.31	16.86	5.52	9.6 ±3.2	12.47 ±1.29	13.0; range 7.8–15.0	dark	sperm, oogonia, oocytes
7/6/64	51.14 ±4.71	32.64	12.28	15.1 ±1.84	10.13 ±1.14	20.3; range 10.4 –45.0	cream, light orange	sperm, oocytes in germinal vesicle stage
7/20/64	56.33 ±4.16	37.69	16.53	15.1 ±1.86	9.80 ±1.21	45.5; range 20.8–72.8	cream, light orange	sperm, oocytes in germinal vesicle stage
8/4/64	56.20 ±4.20	43.19	17.24	14.6 ±1.30	8.50 ±1.00	53.4; range 20.8–72.8	cream, orange	sperm, oocytes with orange pigmentation
8/20/64	59.36 ±2.09	49.99	21.01	16.66 ±4.41	9.93 ±1.10	59.5; range 20.8–91.0	cream, orange	sperm, oocytes with germinal vesicle shrinkage
9/4/64	61.39 ±3.74	52.85	22.02	15.52 ±2.6	11.41 ±0.9	58.5; range 26.0–104.0	cream, orange	sperm, oocytes with germinal vesicle breakdown, few eggs
9/20/64	64.85 ±3.36	63.29	26.95	20.95 ±2.9	10.26 ±1.40	67.6; range 20.8–104.0	cream, bright orange	sperm, eggs, oocytes with germinal vesicle breakdown
10/4/64	65.39 ±1.39	64.77	23.08	15.9 ±2.9	11.46 ±1.47	49.38; range 20.8–98.8	cream, pale orange	sperm, few eggs, oocytes in germinal vesicle stage
10/20/64	63.00 ±2.98	54.25	17.31	17.31 ±1.8	12.05 ±1.08	50.31; range 18.2–104.0	cream, pale orange	sperm, few eggs, oocytes with germinal vesicle breakdown
11/20/64	64.75 ±4.09	63.04	26.22	9.46 ±2.5	14.2 ±0.85	—	pale brown	residual sperm, neutral ovary

the middle of June and a reciprocal relationship was maintained until the end of the reproductive period. An increasing trend in the digestive gland index was observed beginning from the spawning period and it reached a higher value than gonad index by the later part of November.

Changes in gonad coloration

The changes in gonad coloration during reproductive period are as described previously for this species (Sastry, 1963). The entire gonad was covered with a dark peripheral membrane at the beginning of reproductive period. As the gonads begin to differentiate into proximal testicular and distal ovarian regions, a demarcating line separated the two portions. The dark membrane was lost and a cream color in the testis and orange color in the ovaries appeared by the time the gonads reached maturity. The coloration of gonads was gradually lost with the onset of spawning and they were pale brown in spent animals.

Changes in microscopical condition of gonads

The microscopical changes observed during the reproductive period, from May through November, are listed in Table I. The gross microscopical changes of gonads in this species closely follow the descriptions made for other hermaphroditic pectens, by Dakin (1909), Mason (1958) and Reddiah (1962), from detailed histological studies. In this study, the progressive stages in oocyte development, from primary germ cell to the stage prior to spawning, are described to facilitate understanding of temperature effects to be considered in the following sections of this article.

In May, the testicular region contained a large number of spermatogonia and spermatocytes. From the middle of June until the later part of September, spermatozoa predominated and they were closely packed in the follicles. The testicular region increased with further gonadal growth during this period. Towards the later part of November, only a few spermatozoa were retained.

The process of oocyte development, from primary germ cell to the stage prior to spawning, is similar in many respects to other bivalve molluscs (Raven, 1961). Following the vegetative phase, the gonads contained large amounts of connective tissue and narrow tubules ramified to form indistinct follicles in the ovary. The primary germ cells on the germinal epithelium of the gonad wall develop into oogonia and oocytes (Raven, 1958). The oogonia increased in size, with their free margins protruding into the lumen of the follicles while they were still attached with a stalk to the germinal epithelium. The oocytes detached from the germinal epithelium and began growth by an initial increase in size of the nucleus and nucleolus.

The oocytes in the early stage of development contained a large germinal vesicle surrounded by a thin layer of cytoplasm. The oocytes passed through a rapid growth phase by a simultaneous increase in nuclear and cytoplasmic regions. The cytoplasmic granules were transparent in spherical oocytes of this stage. Following the synchronous growth phase, orange pigmentation appeared in the cytoplasm. Beginning with the appearance of orange pigmentation, the cytoplasmic region increased while the germinal vesicle remained about the same size. Maturation process commenced with an initial shrinkage and later disappearance of the germinal vesicle. Immediately after germinal vesicle breakdown, the pigmented cytoplasmic granules were uniformly distributed in the entire oocyte. The oocytes were of maximum size at this stage (80–104 μ diameter). The diameter of the oocytes decreased and the cytoplasmic granules were closely packed, leaving a clear space between the egg surface and the outer membrane (jelly hull, Costello *et. al.*, 1957). The eggs (average diameter 63 μ) released in this stage completed maturation on fertilization, external to the parent animal.

The changes in average oocyte size during the reproductive period are shown in Figure 2. Few oogonia were present in May. By the middle of June, a large number of oogonia and few oocytes were observed. The oocytes were in the beginning of synchronous growth phase (average diameter, 20 μ) by early July. Towards the later part of July, the oocytes were about 40–45 μ in diameter and some of them began to show orange pigmentation. The pigmented oocytes predominated in early August and germinal vesicle shrinkage was noted by the later part of the month. In early September, few eggs were observed and their number increased towards the end of this month. Although the reproductive condition of each collection was fairly uniform, there was variation in size and exact stage of oocytes in different follicles of the same gonad. While eggs predominated in late September, earlier stage oocytes were also present. Animals examined in

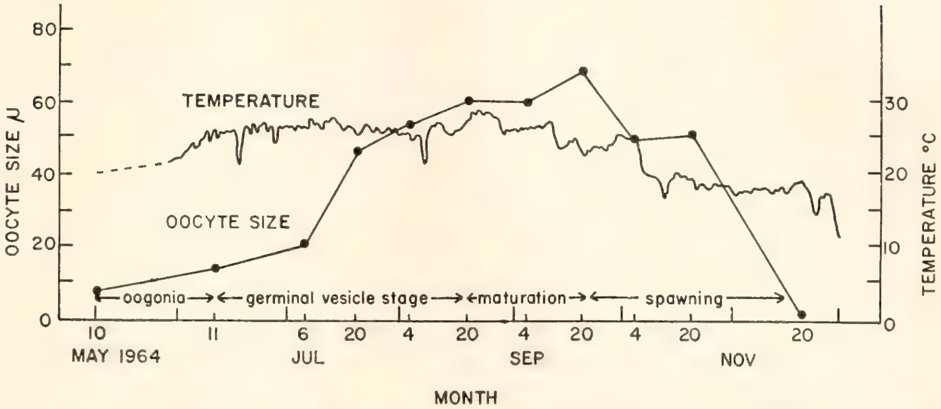

FIGURE 2. Changes in average oocyte size of scallops collected at intervals during the reproductive period. Reproductive condition of the population is shown on the bottom of the graph. The solid line shows the daily noon-time sea water temperature observed in the vicinity where scallops have been collected.

October showed few eggs and oocytes. The gonads decreased in size by the later part of November and the ovaries were empty (Fig. 2).

Changes in environmental temperature during the reproductive period

The daily noon-time water temperatures observed in the vicinity where scallops were collected are shown in Figure 2. The temperature began to increase from about 20° C. in early May to 25° C. by the middle of June. The temperature remained above 25° C. from the middle of June to the middle of September, except for occasional fluctuations. From the middle of September, the temperature began to decrease and it varied between 20° and 25° C. until the beginning of October. The temperature was below 20° C. in early October, and it fluctuated around 17°–18° C. until the later part of November.

Availability of food in scallop habitat

The bay scallops feed on microflora consisting of benthic and tychopelagic diatoms, detritus and bacteria, by filtering the suspended food in the water (Davis and Marshall, 1961). In the shallow estuarine habitat of scallops, the tidal currents continuously stir the sea water and keep the phytoplankton and detritus in suspension. Williams and Murdoch (in press) estimated the phytoplankton production for the same area where scallops have been collected and found the highest production in summer and early fall, and low values throughout the rest of the year (Fig. 3). The phytoplankton production had a pronounced seasonal cycle closely following the temperature cycle. The phytoplankton was found to be chiefly nannoplankton (Williams and Murdoch, in press).

The bay scallops have a relatively high rate of water filtration, probably correlated with their active mode of life and rapid growth rate (Chipman and Hopkins, 1954). The rapid growth of scallops and their gonad development

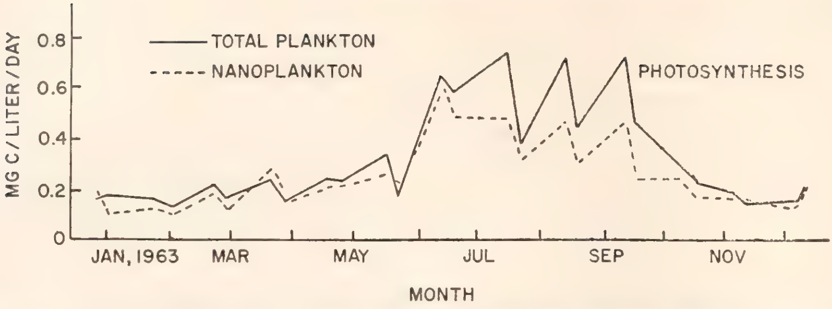

FIGURE 3. The phytoplankton production in the Beaufort Channel, North Carolina. The scallops were collected in the same locality. (The results on phytoplankton production are being published by Dr. Richard Williams, Radiobiological Laboratory, U. S. Fish and Wildlife Service, in a separate paper. I am indebted to Dr. Williams for permitting me to quote his results here.)

(Table I) appear to follow closely the increasing temperature and phytoplankton production beginning in May.

EXPERIMENTAL RESULTS

The groups of animals maintained at various temperatures are shown in Table II. The gonad index, digestive gland index and the microscopical condition of the sample analyzed from the same collection to which these temperature groups belonged, provided the basis for assessing the temperature effects in reproductive period (Table I).

Temperature effects on gonad growth and gametogenesis (Group I)

Temperature effects on group I are shown in Table III. The gonad index and digestive gland index decreased at all the temperatures, but their decrease was more rapid at higher temperatures. The gonads of animals at 10° C. were whitish and spermatocytes were observed in biopsies from testis and ovary. Spermatocytes were observed in gonadal biopsies of animals at 20° C. and 30° C. Oogonia and oocytes were absent. At these temperatures the gonads were translucent, indicating

TABLE II

Groups of scallops collected at intervals during the reproductive period and maintained at different temperatures

Group	Date collected and introduced to experimental temperatures	Experimental temperatures ± 0.5–1.0° C.	Total number of animals	Size mm. mean ±S.D.
I	6/11/64	10, 20, 30	24	35.13 ± 3.28
II	7/6/64	10, 15, 20, 25, 30	50	52.28 ± 3.21
III	8/20/64	10, 15, 20, 25, 30	50	59.24 ± 3.92
IV	10/20/64	10, 20, 25, 30	24	62.06 ± 2.62
V	11/20/64	10, 15, 20, 25, 30	30	65.13 ± 3.55

TABLE III

Temperature effects on scallops during gonad growth and gametogenesis (Group I)

Temperature ° C.	No. animals	Average size mm.	Average weight g.	At the time of 50% survival					
				Average gonad index	Average digestive gland index	Spawned	Gonad condition		
							Average oocyte diameter μ	Color	Microscopical
10	8	34.5	12.81	3.3	10.64	—	absorbed	whitish	spermatocytes
20	8	35.6	14.12	3.2	4.96	—	absorbed	pale brown	spermatocytes
30	8	35.2	13.50	3.2	3.70	—	absorbed	pale brown	spermatocytes

that the gonadal tissue was utilized for maintenance of the animals. The spermatocytes, predominant at 10° C., were reduced in number at higher temperatures.

Temperature effects on animals with accumulated gonad reserves (Group II)

The gonad index of the sample analyzed before introducing this group to experimental temperatures was three times greater than in May and the oocytes were in the beginning of growth phase (Table I). Temperature effects on this group are shown in Table IV. There was a slight decrease in gonad index and

TABLE IV

Temperature effects on scallops with accumulated gonad reserves and oocytes in the beginning of growth phase (Group II)

Temperature ° C.	No. animals	Average size mm.	Average weight g.	At the time of 50% survival					
				Average gonad index	Average digestive gland index	Spawned	Gonad condition		
							Average oocyte μ	Color	Microscopical
10	10	53.4	51.6	14.2	9.3	—	23.0	cream, pale orange	sperm, oocytes
15	10	53.0	41.6	11.1	5.1	—	absorbed	pale brown	spermatocytes
20	10	53.1	38.0	6.0	9.1	—	absorbed	pale brown	spermatocytes
25	10	48.6	34.0	6.9	7.8	8th day sperm, eggs	—	pale brown	residual sperm
30	10	53.3	39.6	10.3	8.1	5th day sperm, eggs	—	pale brown	residual sperm

digestive gland index at 10° C.; however, the oocytes remained in the beginning of growth phase (average diameter, 23 μ). Spermatozoa were retained in the testicular region. The gonad and digestive gland indices decreased at 15° C. and 20° C. On examination of gonadal biopsies, absence of oogonia and oocytes and presence of spermatocytes and spermatozoa were observed.

Eggs and spermatozoa were released at 25° C. and 30° C. on the eighth and fifth day, respectively. On fertilization, the eggs developed to normal larvae, being maintained at the temperature they were released. Apparently, the decrease in gonad index of these animals was partially due to loss of gametes from the gonads.

Effect of temperature on animals with oocytes in the beginning of maturation (Group III)

Table V shows the temperature effects on group III. The temperature effects were similar to those observed for the previous group with two notable exceptions. In addition to the animals at 25° C. and 30° C., those maintained at 20° C. also released gametes. The time for release of gametes in this group was less than that for the previous group (Fig. 4). The gonad index values and observation of gonadal biopsies indicated that they had completely discharged the gametes.

At 10° C. there was no appreciable change in either gonad index or digestive gland index, but the oocytes were observed to disintegrate by rupture of the

TABLE V

Temperature effects on scallops with accumulated gonad reserves and oocytes in the beginning of maturation (Group III)

Temperature °C.	No. animals	Average size mm.	Average weight g.	At the time of 50% survival					
				Average gonad index	Average digestive gland index	Spawned	Gonad condition		
							Average oocyte diameter μ	Color	Microscopical
10	10	58.49	50.85	16.7	8.9	—	57.2	whitish pale orange	mature sperm
15	10	58.64	53.36	14.54	10.24	—	—	cream, pale orange	mature sperm, eggs with ruptured membrane
20	10	59.31	54.46	7.73	9.64	10th day sperm, eggs	—	pale brown	residual sperm
25	10	59.68	56.46	8.20	9.8	6th day sperm, eggs	—	pale brown	residual sperm
30	10	60.08	57.41	9.81	8.4	4th day sperm, eggs	—	pale brown	residual sperm

surrounding membrane. Oocytes in earlier stages of development remained in about the same condition. Spermatozoa were observed in large numbers in the peripheral tissue of the ovary. At 15° C. spermatozoa and disintegrating oocytes were observed in the gonads. The gonad index changed very slightly. Loosanoff and Davis (1951) observed reabsorption of ova, when spawning was delayed by maintaining them at low temperatures.

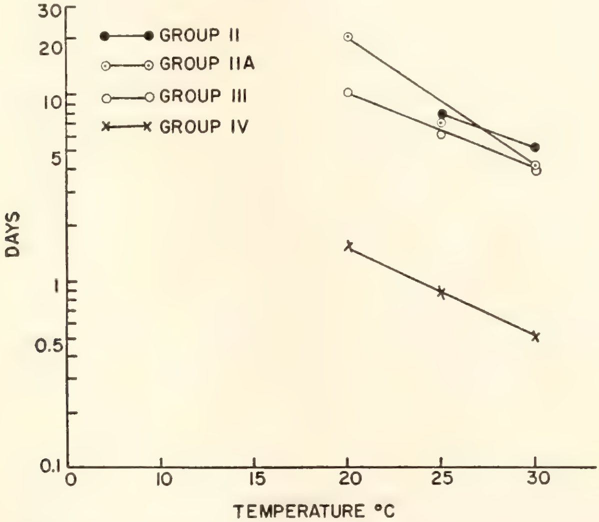

FIGURE 4. The time taken for spawning of scallops at different temperatures in the laboratory. Each group represents a collection made at intervals during the reproductive period. Group IIA is a collection made on July 20, 1964. Note the time-temperature relationship for spawning in different groups.

During September, when the animals were in mature physiological state, they readily liberated gametes even before the sea water in the containers reached the desired experimental temperature.

Temperature effects on animals with partially spent gonads (Group IV)

This group was collected after spawning had commenced in the field population. The results, shown in Table VI, indicate that the temperature effects were similar to those observed for the previous group. The gametes were released at 20°, 25°, and 30° C. The time taken for spawning at these temperatures was less than that for the previous group (Fig. 4). Residual spermatozoa and a few early-stage oocytes were observed on examination of gonadal biopsies. On fertilization, the eggs developed to normal larvae at the temperature they have been released. The animals at 10° C. remained in the same condition as when introduced.

Effect of temperature on animals with spent gonads (Group V)

Table VII shows the temperature effects on group V. The digestive gland index of animals at 10° C. and 15° C. decreased slightly, but the gonad index

TABLE VI

Effect of temperature on scallops with gonads in spawning condition (Group IV)

Temperature ° C.	No. animals	Average size mm.	Average weight g.	At the time of 50% survival					
				Average gonad index	Average digestive gland index	Spawned	Gonad condition		
							Average oocyte diameter μ	Color	Microscopical
10	6	62.4	60.89	20.1	12.35	—	51.06	cream, orange	sperm, oocytes and eggs
20	6	62.2	58.46	10.24	13.32	about 36 hours sperm, eggs	—	pale brown	residual sperm
25	6	62.2	63.32	13.42	11.81	12–30 hours sperm, eggs	—	pale brown	residual sperm
30	6	61.4	59.86	14.40	10.64	about 12 hours sperm, eggs	—	pale brown	residual sperm

remained approximately the same. Residual spermatozoa and a large number of spermatocytes were observed in the testicular region, whereas the ovarian region remained neutral without formation of follicles or oogonia. At 20° C., the gonad index decreased but the digestive gland index remained about the same.

TABLE VII

Temperature effects on scallops with neutral gonads (Group V)

Temperature ° C.	No. animals	Average size mm.	Average weight g.	At the time of 50% survival					
				Average gonad index	Average digestive gland index	Spawned	Gonad condition		
							Average oocyte diameter μ	Color	Microscopical
10	6	65.5	69.39	8.57	12.57	—	—	pale brown	spermatocytes
15	6	64.0	64.52	9.17	11.00	—	—	pale brown	spermatocytes
20	6	64.5	67.26	6.52	11.58	—	—	pale brown	spermatocytes
25	6	65.7	65.96	5.58	6.30	—	—	pale brown	spermatocytes
30	6	66.1	69.29	4.92	8.45	—	—	pale brown	spermatocytes

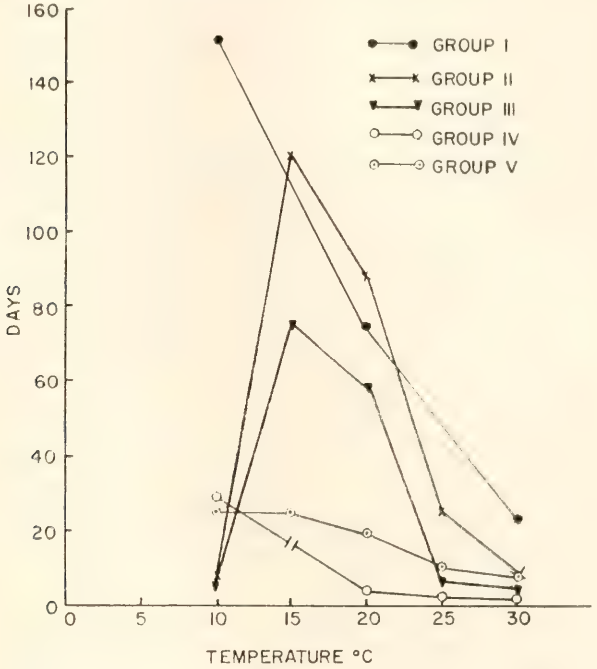

FIGURE 5. The time for 50% survival of scallops maintained at different temperatures. Each group represents a collection made at intervals during the reproductive period.

Some animals at this temperature formed indistinct follicles and a few oogonia. The digestive gland and gonad indices of the animals at 25° C. and 30° C. decreased and no gametogenesis was initiated.

Temperature tolerance of scallops during reproductive period

The time for 50% survival of different groups maintained at various temperatures is shown in Figure 5. Group I tolerated all the temperatures longer than any other group maintained under similar conditions. The tolerance of scallops to 10° C. decreased considerably when the oocytes were developing. Animals in spawning condition were least tolerant to all the temperatures, but those maintained at 20° C. and above released gametes before death. The animals with neutral gonads survived slightly longer than the spawning animals. Temperatures between 15° C. and 25° C. seem to be better suited for scallops in reproductive condition.

DISCUSSION

The reproductive cycles of marine invertebrates include several events: activation of gonad growth following the vegetative phase, gametogenesis, maturation, spawning and the resting stage (Giese, 1959). The suggestion that temperature regulates breeding period (Orton, 1920; Runnström, 1927) and the possible genetic control of differences in breeding temperatures of latitudinally separated populations of a species (Korringa, 1957) are based on only one event in the reproductive cycle. The results of this study show that temperature influences the events in the reproductive period beginning with gonad growth.

Temperature effects on gonad growth

The scallops exposed to various temperatures during the period of gonad growth (group I), without food supply, showed a decrease in both gonad and digestive gland indices. Although the digestive gland index was greater than the gonad index before this group was introduced to the experimental temperatures, no gonad development was observed at any of the temperatures. This shows that additional food supply is essential for gonad growth and that the reserves accumulated in the digestive gland are not sufficient to support this process. The review of Giese (1959) cites some examples of marine invertebrates in which gonad tissue has been utilized when they were starved long before the breeding period. Barnes, Barnes and Finlayson (1963) point out that food is stored in the boreo-arctic species of barnacles when it is most abundant and utilized for gonad development later in the season, thus ensuring the availability of food during the period of gonad development. It is possible that different species might have adapted differently for nutrient storage and utilization in gonad development.

In the scallops beginning their first reproductive period, growth and gonad development take place simultaneously (Table I), indicating that the ingested food might be rapidly digested and utilized. The mechanism of nutrient transfer to gonads has been considered to be either through blood circulation or direct transfer (Barnes, Barnes and Finlayson, 1963; Lawrence, Lawrence and Giese, 1965). A reciprocal relationship between the gonad index and the digestive gland index during the reproductive period has been reported in *Pisaster ochraceus* (Farmanfarmaian *et al.,* 1958) and *Haliotis cracherodii* (Boolootian *et al.,* 1962). The reciprocal relationship between the gonad and the digestive gland indices of scallops during the reproductive period indicates that the nutrients taken up by the animals might be rapidly utilized for growth and development, without prior storage. It is likely that all the nutrients taken up by the animals might be rapidly utilized and hence a low digestive gland index might result during the reproductive period. The digestive gland index of scallops in the resting stage was higher than the gonad index and this period coincides with decreased environmental temperature and reproductive inactivity. The gonads did not develop when the resting stage animals (immediately after completion of spawning in the field) were maintained at various temperatures without food supply. The gonad index of animals maintained at lower temperature remained the same, whereas the digestive gland and gonad indices decreased at higher temperatures. It appears that all the reserves available in the animals have been utilized for maintenance at higher temperatures. Therefore, it is possible that gonad growth might take place in the presence of abundant food and under temperature conditions at which nutrient mobilization to gonads is permitted after the metabolic needs of the animals have been met.

Temperature effects on gametogenesis, maturation and spawning

Although gametogenesis is initiated when the gonads have a minimum amount of reserves (*i.e.,* May and June; Table I), a continued transfer of nutrient reserves to the gonads appears to be necessary for further growth and development of gametes (Table III). Various biochemical reserves are mobilized to the gonad synthetic centers when gametes are formed (Giese, 1959; Barnes, Barnes and Finlayson, 1963). Starvation of scallops during the period of gonad growth resulted in an absorption of oogonia and oocytes at all the temperatures. The temperature effects on spermatogenesis were not so pronounced. Spermatocytes were present in the testis and the peripheral tissue of the ovary of animals maintained at all temperatures but they failed to mature (Tables III and VII).

The animals with accumulated gonad reserves (Tables IV–VI) when maintained at 20° C. and above released gametes, even though they were starved. This shows that once a certain amount of reserves is present in the gonads and if the temperatures are suitable for gamete growth to maturity and spawning, these processes might be irreversible. The development of eggs seems to proceed normally without making the reserves available for maintenance of the starving animals. The time at which food is withheld might be critical in gonad growth and gamete development.

When the oocytes are in the beginning of growth phase (Table IV), the animals maintained at 20° C. and below failed to mature and spawn. However, when the oocytes are in the later stages of development, the animals maintained at 20° C. also spawned, but those held at 15° C. and 10° C. failed to spawn. The scallops seem to require temperatures slightly above 20° C. for development of oocytes to the stage prior to release. The temperatures at which spawning occurred decreased the further the oocytes had developed toward maturity. The spawning of the laboratory-maintained animals was ahead of those in the field (Fig. 4, Groups II and III). Crisp (1957) reported that starvation accelerates maturation of barnacles, provided the eggs are developing in the ovary.

The time-temperature relation for spawning of scallops from different collections (Fig. 4) indicates that the development of oocytes to maturity might be a function of temperature within a range characteristic for the species, if the gonads have already accumulated sufficient reserves to support the synthetic activities of the developing gametes. Loosanoff and Davis (1952) showed that the time required for maturation of oysters, *Crassostrea virginica,* is dependent on the maintenance temperature. They also found that oysters with poor glycogen reserves failed to reach maturity. The later stages in gonad development of scallops (*i.e.,* oocyte growth, maturation and spawning) might be influenced by maintaining the animals at optimum temperatures, but the success of these processes appears to depend on the presence of reserves in the gonads. Hence, any factors affecting food collection, or nutrient mobilization to gonads would also affect the reproductive activities of the field population.

Ecological considerations

The reproductive period of the scallops is regulated to the warm temperatures of summer and early fall. The laboratory results on temperature effects on reproduction suggest that the warm temperatures of summer are necessary for oocytes to develop to the stage prior to release. The gonad growth is timed to a period when phytoplankton is most abundant in the environment and this seems to provide enough food for the animals to accumulate reserves in the body for maintenance and for mobilization to the gonads. The abundance of food has been generally associated with breeding period of marine invertebrates and thought to ensure adequate nutritional availability for the planktonotrophic larvae (see Thorson, 1950; Giese, 1959; Boolootian, 1964, for discussion), but the factors responsible for correlating these events are not clear. Although synchronization of spawning with the period when food is most abundant for larvae is important, the adults also seem to require large amounts of food for gonad growth. A decrease in phytoplankton production during peak spawning period could be seen in Figure 3. The food required for larvae and the adults might be different in their quality and quantity. Sastry (1965) reared the scallop larvae to preadults by providing unicellular algae as food. Additional experiments to determine the value of different organisms in plankton as food for larvae are needed for an understanding of the relationship between food and reproduction.

In general, on the days when spawning occurred in the laboratory animals, it was observed to take place when they were changed to fresh sea water of equilibrated temperatures. Although care was taken, slight changes in temperature and disturbance of animals could not be avoided. It is likely that once the animals have reached a mature physiological state, a slight change in any of the responsible factors might induce a spawning rhythm. Sastry (1963) reported stimulation of spawning in scallops by temperature changes and indicated that spawning might be dependent on the maturation condition of gonads and a favorable stimulus. The lower temperature limits for early cleavages of fertilized scallop eggs have been found to be between 15° C. and 20° C. (unpublished data). The timing of spawning to the period when temperatures are suitable for completion of early stages in development would also be an important factor in regulating the breeding period. Runnström (1936) determined the temperature limits for egg development in a number of marine invertebrates, and Thorson (1950) pointed out that the temperatures during breeding period were within the lethal limits for development of eggs and larvae of a species that can spawn within a given area.

In conclusion, the events in the reproductive period of scallops appear to have been regulated to the period when phytoplankton is most abundant and temperatures are favorable for gonad development. The duration of these two factors in the environment might determine the length of gonad activity. Further experiments are needed to understand the phasing of these events in the reproductive period and the factors responsible for timing of breeding period in nature. Barnes (1963) demonstrated the interaction of light and temperature in gonad development and maturation of barnacles. Boolootian (1963) reported that the testes of *Strongylocentrotus purpuratus* responded differently by production of gonia cells at long-day stimulus, and spermatocytes, spermatids and spermatozoa at reduced day-length. The influence of light and temperature and their mutual interaction on different events in reproduction should be carefully analyzed to determine environmental control of annual reproductive pattern of marine invertebrates.

SUMMARY

1. The events in the reproductive period of a population of bay scallops, *Aequipecten irradians,* have been analyzed from the beginning of gonad growth to the resting or neutral stage. The gonad index of field animals increased rapidly

between May and early July, and the population reached a physiological state to commence spawning towards the later part of September. The spawning period covered approximately two months between September and November.

2. Gonad growth coincides with increasing temperature and peak phytoplankton production in early summer. The gonad index was maximum when the population was ready to commence spawning. The gonad index and the digestive gland index showed a reciprocal relationship during the reproductive period.

3. Scallops collected at intervals during the reproductive period were maintained at various temperatures in the laboratory, without food. The temperature effects on the events in the reproductive period have been studied.

4. Maintenance of scallops at various temperatures during the period of gonad growth resulted in a decrease in digestive gland and gonad indices. The oogonia and the oocytes were absorbed. The spermatocytes in the testis failed to mature.

5. Scallops with accumulated gonad reserves and developing oocytes in the ovary, when maintained at 20°, 25° and 30° C. rapidly matured and spawned. The scallops maintained at 10° C. and 15° C. failed to mature and spawn.

6. When the gonads were neutral, maintenance of animals at any of the experimental temperatures did not permit increase in gonad index or initiation of oogenesis. These animals had a high digestive gland index before they were introduced to the experimental temperatures.

7. The scallops with developing oocytes in the ovary, when maintained at 20°, 25° and 30° C. in the laboratory, released gametes much earlier than the field population. The time required for release of gametes showed a direct relationship to temperature between 20° C. and 30° C.

8. The scallops maintained at various temperatures in the beginning of gonadal growth and gametogenesis survived longer than any other group maintained under similar conditions during the reproductive period. The animals in spawning condition were least tolerant to all the temperatures.

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A STUDY OF THE TERMINAL INNERVATION OF A FAST-ACTING FISH MUSCLE

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This study is concerned with the investigation of the morphology and distribution of the endplates in the intrinsic striated muscle in the wall of the swimbladder of the toadfish, *Opsanus tau*. Recently Skoglund (1959) showed that swimbladder muscle reaches its contraction peak within 5–8 msec. and relaxation is completed in an additional 5–7 msec. Fawcett and Revel (1961) observed by electron microscope studies of the muscle fiber the unique shape and radical disposition of the myofibrils, as well as an elaborate branched sarcoplasmic reticulum. They reported this reticulum has large transverse triads which might indicate an efficient system for intracellular impulse conduction. Because of its unusual morphological and physiological properties, it was thought an understanding of the terminal innervation of this muscle might be of value to future investigators.

Materials and Methods

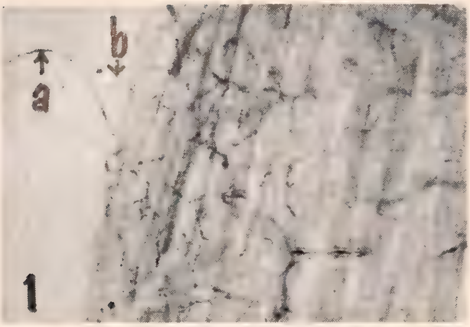
The experiments were performed on the common toadfish, *Opsanus tau*. Their ages, approximated from the length and sex of the fish (Schwartz, 1963), ranged from less than one to almost eleven years. Animals of both sexes were used. In all experiments the animal was immobilized with a 0.01% (W/V) solution of MS-222 Sandoz in sea water.

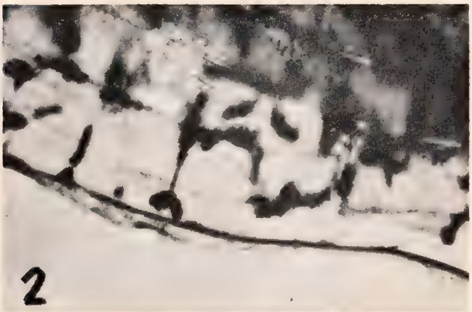
Methylene blue perfusion, gold chloride toning and cholinesterase localization were selected to stain the peripheral nerves and their endings.

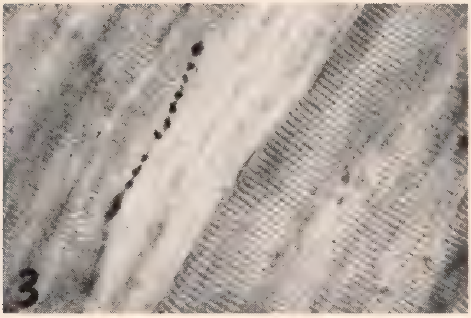
Intravital staining was accomplished by a methylene blue perfusion technique. The staining fluid was prepared a few hours before use and contained: Methylene blue (Allied Chemical C.I. 52015, Cert. No. 400) 0.25 g., Resorcin (Merck) 0.15 g., NaCl 8.00 g., phosphate buffer (pH 5.4–6.2) 10.00 ml., distilled water 1000.00 ml.

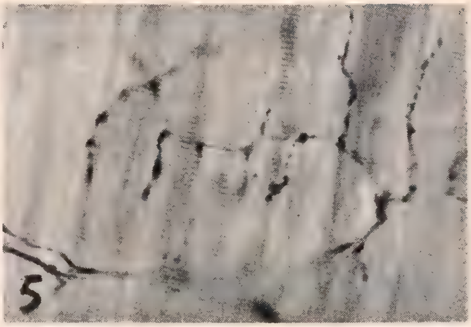
Oxygen was bubbled through the stain solution throughout the procedure. A three-way valve enabled the stain to be drawn into a 50-ml. syringe and then forced through the tubing cannulate while maintaining airtight connections. A midventral incision was made the length of the fish and both the heart and swimbladder were exposed. Extreme care was taken to avoid severing large blood vessels. Two small slits were made in the conus arteriosus, one for the insertion of the cannula and the other for exsanguination. Once inserted the stain was forced through the cannula. When air bubbles were noted the cannula was removed, cleared of the obstruction and then reinserted. A volume of about 150 ml. was injected in 10–15 minutes and no longer than 20 minutes.

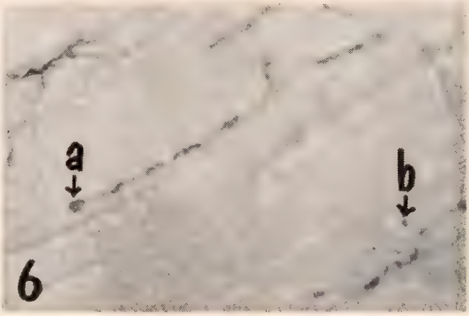
Following perfusion small pieces of muscle were removed and placed on loosely piled gauze, allowing full exposure to the air. The tissue was then saturated with staining fluid and after about 20 minutes it acquired a deep blue shade. The

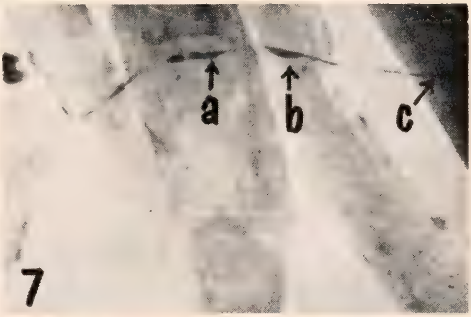












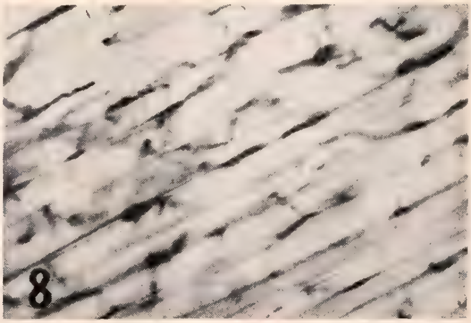


FIGURE 1. This and succeeding figures are of teased toadfish swimbladder muscle. Intramuscular nerve plexus showing terminal innervation. (a) Axionic swelling on preterminal nerve fiber. (b) Single axonic collateral branch with verrucose ending. Methylene blue. × 300.

stained tissue was immediately fixed in chilled 8% ammonium molybdate in 0.8% saline, refrigerated (0–3° C.) overnight, and subsequently washed in several changes of tap water, for a total of two hours.

Dehydration of the tissue was initiated in 95% ethanol for 15 minutes, followed by changes of absolute ethanol and xylene for 15 minutes each. After teasing the pieces into their component fascicles, they were mounted in Permount.

The gold chloride technique used was Cole's (1946) modification of Ranvier's original staining method. Following this treatment muscle fascicles were teased and mounted in glycerine for study.

For acetylcholinesterase localization the technique of Koelle (1951) was employed. Teased preparations were incubated 30 minutes to 60 minutes in acetylthiocholine iodide (AThCh), after treatment with $1.5 \times 10^{[illegible]}$ *M* diisopropylflurophosphate (DFP), for 30 minutes. Following this treatment the tissues were washed, dehydrated and mounted in Permount.

RESULTS

The intrinsic muscle of the swimbladder consists of two muscle bands composed of short fibers running transversely to the long axis of the muscle. The muscle fibers ranged from 30 μ to 50 μ in diameter.

Neural elements were interpreted in swimbladder muscle by integrating the results of the three staining methods employed in this study. The motor nerves innervating the swimbladder muscle are composed of single fascicles enclosed in perineural sheaths. The nerve fibers within the fascicles appear to be myelinated and are of relatively consistent diameter, ranging from 7 μ to 11 μ. The main nerve trunk passed at right angles to the direction of the muscle fibers, while preterminal fibers branched from the main trunks either in small groups or singly (Figs. 1, 2, 5) and in most cases ran parallel to the long axis of the muscle fibers (Figs. 3, 5, 6).

The preterminal fibers may end in a single endplate (Figs. 2, 3, 4, 6) or unbranched collaterals terminating in bead-like dilatations (Figs. 3, 5). Frequently the marginal, longitudinal, preterminal fibers give off collateral branches which cross the muscle fibers at right angles and terminate marginally on the opposite side of the muscle fiber in club-like expansions or verrucose dilatations about 2 μ in diameter (Figs. 1, 6, 8).

The length of the endplates ranged from 2 μ to 100 μ for the most diffuse type of ending and adjacent endings on the same muscle fiber ranged from 20 μ to 80 μ apart.

FIGURE 2. Motor endplates and terminal nerve twigs stained for AChE localization. × 1000.

FIGURE 3. Bead-like ending with enlarged terminus. Methylene blue. × 450.

FIGURE 4. Isolated muscle fiber showing four junctional areas. AChE localization. × 100.

FIGURE 5. Preterminal nerve fiber showing diffuse terminal innervation. Methylene blue. × 300.

FIGURE 6. (a) Shows a discrete ending on a muscle fiber. (b) Shows a single axonic collateral branch with verrucose ending. Methylene blue. × 300.

FIGURE 7. Preterminal nerve fiber with axonic swellings indicated by arrows. Gold chloride. × 1200.

FIGURE 8. Acetylcholinesterase localization, showing a variety of neural elements. The darker stained elements terminate the lighter stained preterminal fibers. × 300.

Discrete axonic swellings were seen in terminal myelinated axons stained with all three techniques employed (Figs. 1, 7). Gerebtzoff (1959) reported axonic swellings in the lamprey. The axonic swellings in the toadfish ranged from 9 μ to 25 μ in length and were three to five times wider than the nerve fiber on which they were located. Although the structure of the swellings could not be determined with light microscopy, they stained selectively with Koelle's (1951) histochemical method.

The results of the acetylcholinesterase preparations are shown in Figures 2, 4, and 8. The acetylcholinesteratic activity of the terminal nerve pattern is shown in Figure 8.

In all teased preparations the plexiform pattern of innervation was observed to extend throughout the length of the muscle fiber. It was not possible to determine whether multiple nerve terminations were from the same or different nerve fibers.

Discussion

The results of the present study indicate that there is a variety of terminal nerve elements in swimbladder muscle. Generally, small bundles of nerve fibers tend to intertwine into an intricate plexiform pattern from which the individual motor fibers then separate out into small sprays of indiscrete and discrete endings.

A plexiform pattern of the intramuscular nerve bundles has been cited by Tiegs (1953) in striated muscle of the rabbit and monkey, by Hinsey (1934) in the sartorius muscle of the common frog, and by Cole (1955) and Barets (1955) in the lateral profundus muscle of various fishes.

Sensory endings rarely stain for cholinesterase (Coërs and Woolf, 1959), and only after a prolonged incubation of five hours or more can any faint enzyme activity be detected in them (Coupland and Holmes, 1957; Gerebtzoff, 1959). In addition, the "terminaisons en panier," originally described by Giacomini (Hinsey, 1934) as sensory elements in the fish, have been interpreted as a plexus of motor nerves innervating fast-acting fish muscle (Barets, 1955; Barets and Le Toure, 1956). This corresponds to Tieg's (1953) statement that muscle spindles are absent in fish.

The pattern in extremely rapid extraocular muscles is somewhat similar to that of intrafusal muscle fibers. In addition to multiple equatorial endplates, motor nerve fibers appear to give rise to numerous small branches which innervate series of minute endplate-like structures along the length of the muscle fiber (Gerebtzoff, 1959; Kupfer, 1960). Multiple innervation has been reported for other mammalian muscle fibers (Feindel *et al.*, 1952; Hunt and Kuffler, 1954; Rossi and Cortesina, 1965; Mahran and Sakla, 1965) and occurs in many avian muscles (Ginsborg, 1960; Ginsborg and Mackay, 1961).

Hoyle (1957) examined the twitch muscle fibers of the locust, which structurally resemble the muscle fibers in toadfish swimbladder muscle. He states that multiple innervation might permit impulses to reach the termination at several points along the muscle fiber in less time than permitted by a single myoneural junction. Wiersma (1957) states that in arthopods the nerve fiber itself is often responsible for the conduction of the impulse along the fiber rather than the propagated depolarization of the sarcolemma.

According to Hess (1962, 1963), multiple endings along a single fiber may vary somewhat in form but never to the extent that one fiber may have classically-defined "en grappe" and "en plaque" forms. Garvin (1925) refrained from classifying the diverse endplates in the panniculus carnosus of the hedgehog as either "en grappe" or "en plaque." Wilkinson (1929) saw a similar variation of the endings occurring on different muscle fibers in several species of lizards and frogs. He found different forms terminating the branches of the same motor neuron and associated diffuse endings with smaller fibers and plate-like endings with muscle fibers of larger diameter. He suggested that the bead-like endings of the terminal axons represented growing ends of nerve and that the grape-like endings are a developmental form, both ontogenetically and phylogenetically. More recently, Csillik (1961) proposed that the diffuse ending is a regenerative as well as ontogenetically immature form of the plate-like ending.

Because of the variety of nerve terminals observed in the swimbladder muscle, it was difficult to classify them specifically. Studies are in progress at the present time to attempt to solve this discrepancy.

SUMMARY

1. Histochemical localization of the enzyme, acetylcholinesterase (AChE), methylene blue perfusion, and gold chloride were employed in studying the motor nerve innervation of the intrinsic striated muscle located in the wall of the swimbladder of the toadfish, *Opsanus tau*.

2. A plexiform pattern of multiple innervation was revealed.

3. Both preterminal nerve fibers and nerve terminals showed acetylcholinesterase activity.

4. A wide variation of shape, size and distribution of nerve endings was seen. The endplates ranged from 2 μ to 70 μ in length and were spaced from 20 μ to 480 μ along a muscle fiber.

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PHYSIOLOGICAL AND ECOLOGICAL STUDIES ON THE BIVALVE MOLLUSK ARTHRITICA BIFURCA (WEBSTER, 1908) LIVING COMMENSALLY WITH THE TUBICOLOUS POLYCHAETE PECTINARIA AUSTRALIS EHLERS, 1905

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Known commensal bivalves from New Zealand waters are the galeommatid *Scintillona zelandica* (Odhner) described in association with the synaptid holothurian *Trochodota dendyi* by Morton (1957), *Coriareus neozelanicus* Dell (Leptonidae) recorded by Dell (1963) as attached to a new species of the echinoid genus *Cyclaster* in deep water, and two species of the leptonid genus *Arthritica*. *A. crassiformis* Powell is found living with the large rock-boring pholad *Anchomasa similis* (Gray), and *Arthritica hulmei* Ponder lives in association with the sea-mouse *Aphrodita australis* Baird. The biology and commensal relationships of these two species of *Arthritica* have been described by Ponder (1965).

The third New Zealand species of the genus *Arthritica, A. bifurca* (Webster), was considered by Ponder (1965) to be free-living before I noted its association with *Pectinaria australis.*

I would like to thank Professor J. E. Morton and Mr. W. F. Ponder for co-operation in this work, and Drs. R. K. Dell and R. B. Pike for constructive criticism of the manuscript.

SEASONAL VARIATIONS IN PECTINARIA AUSTRALIS AT PETONE BEACH (Fig. 3)

The tidal rise and fall at Petone Beach is 4.5 feet (1.37 meters) at spring tides which at low water uncover the sand for a distance of approximately 50 m.

Pectinaria australis is found at the high water neap tide level and occurs down to low water spring tides, but may extend below this level. *P. australis* lives within a conical tube of cemented sand grains in fine muddy sand. The broader anterior end of the tube is directed downwards and the narrow posterior end lies near the surface of the sand (Fig. 1). The location of *P. australis* on the exposed sand surface is usually indicated by a characteristic small exhalant hole one or two millimeters in diameter. In the aquarium, *P. australis* showed no evidence of a secondary inhalant hole in the sand near the tube, as illustrated for *P. koreni* by Watson (1928, p. 31, Fig. 2) but draws the inhalant current from around the sides of its tube as shown in Figure 1.

Samples were taken at low tide during the third week of each month between April, 1964, and March, 1965. Preliminary sampling showed *Pectinaria* to have a very patchy distribution. The position of these patches or "beds" on the beach

Fig. 1

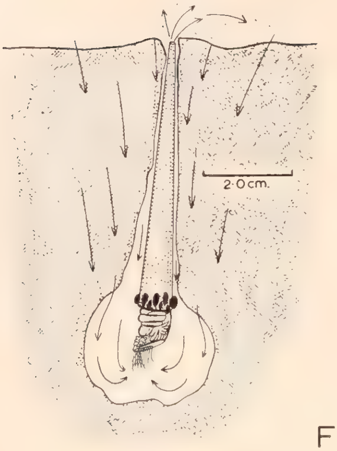

Fig. 2

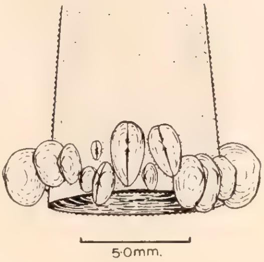

Fig. 3

Seasonal Distribution of *Pectinaria australis* on Petone Beach 1964–65

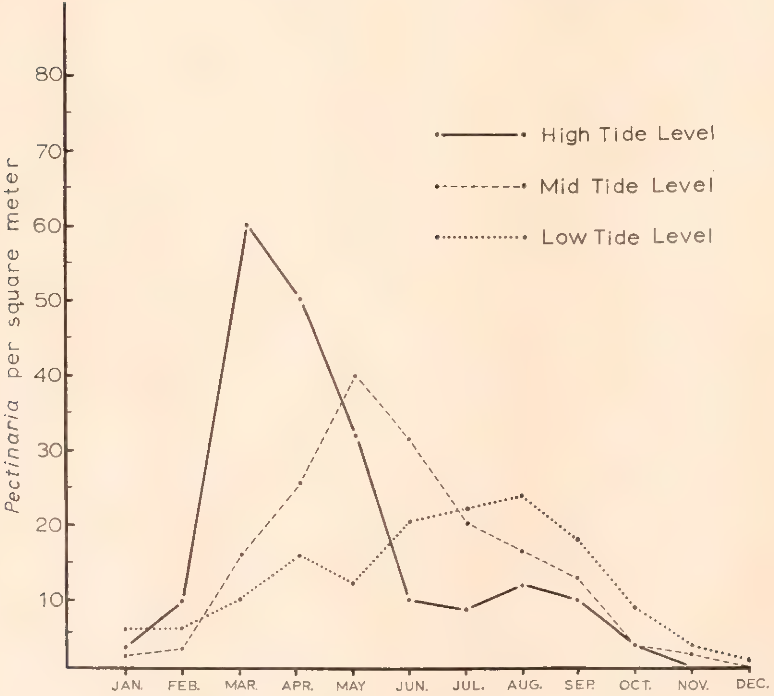

FIGURE 1. *Pectinaria australis* burrowing in aquarium sand. Water currents indicated by arrows. *Arthritica* shaded in solid black.

FIGURE 2. Position and orientation of *Arthritica bifurca* on *Pectinaria* tube.

FIGURE 3. Seasonal distribution of *Pectinaria australis* on Petone Beach. Based on data from samples: April, 1964, to March, 1965.

moved considerably, and transects were made through these areas wherever they occurred. Quadrats of one meter square and about 15 cm. deep were taken at 5-m. intervals from the high water neap tide level down the beach on the selected transects.

The *Pectinaria* population varied in density throughout the year, as shown by Figure 3. The extremely dense population which occurred at high tide level during March and April (autumn) comprised immature worms (tube length 10 mm. to 20 mm.) and appeared to be a separate population from the smaller numbers of much larger adult worms (tube length 35 mm. to 75 mm.) found at lower tide levels during these two months.

In May and June (early winter) the highest densities occurred around mid tide level, and were mainly immature worms (tube length 10 mm. to 35 mm.) while the smaller adult population still occupied the lower tide level. From July through to February the *Pectinaria* community could not be separated into two groups, as all worms were mainly mature and ranged in tube length between 40 mm. and 75 mm. During September and October (spring) the population density at all tide levels fell appreciably until November, December and January, when *Pectinaria* became very rare over the entire beach.

The extremely high numbers of immature worms found during March and April (Fig. 3) suggest that the main larval settlement occurred on the upper beach probably during February. During May and June the upper level population density fell sharply, but the numbers of young worms increased significantly at the mid tide level. As these worms had not increased in length from March they could have been derived from a later settlement and not by downward migration from the upper beach. The less pronounced population increase at low tide level shown during June, July and August could be similarly explained. Thus, the apparent migration of worms down the beach is probably due to mortality among the upshore and earlier settled population rather than to actual movement of the community down the beach.

Hence, with the community decreasing numerically from March through to the following February (Fig. 3) and the worms showing a marked length increase during this time, it is possible that *P. australis* lives for only 12 or 18 months and is subject to an extremely high mortality rate during growth and maturation and also after breeding in the spring. It is also probable that the population is replenished mainly during late summer by settlement of larvae at or near the high tide level, but there is evidence of a fairly extended breeding period.

No study was made of the possible causes for the patchy distribution of *Pectinaria* on Petone Beach. This may be due to gregarious tendencies in the planktonic larvae (Knight-Jones, 1951a, 1951b; Knight-Jones and Stephenson, 1950) or to the patchy occurrence of micro-organic food on the sand grains (Wilson, 1955). Mechanical sand grain analysis similar to the work of Pike and Le Sueur (1958) was not undertaken as there was no visible change in sand grain size in the transect areas.

Association of Arthritica bifurca With Pectinaria australis

Arthritica bifurca is attached by several fine byssus threads to the outer surface of the head-end of the tube of *Pectinaria australis,* orientated vertically with its anterior end directed downwards (Fig. 2). The bivalves were not found inside the tube of the worm and were rarely further than three millimeters from the anterior rim. Two specimens were found, however, attached to the sides of the burrows of *Abarenicola pacifica* (N. Z. lugworm). Ponder (1965) found occasional unattached specimens of *Arthritica bifurca* alive under stones at Auckland, New Zealand. Ponder (personal communication) also found the species living in mud at or below low tide level in the Auckland area where *Pectinaria* occurs, but the bivalves were not observed attached to the worm tubes. However, these samples were collected by dredging and sieving, and the bivalves may have become detached.

From the total numbers of polychaetes and bivalves observed at Petone Beach over the year only 10% of mature worms (tube length greater than 35 mm.) were without *Arthritica bifurca*, and those worms with bivalves carried between one and 22. It was not possible to determine the size range of *A. bifurca* throughout the year or to correlate the size of worm tube with the number of associated bivalves at different tide levels and seasons, owing to extreme variation in these factors—as found by Ponder (1965) in *A. crassiformis*. However, more *Arthritica* were associated with each worm, together with a higher incidence of association on the upper shore, than at lower tide levels.

The color of *Arthritica* changed progressively from creamy-white below the mid tide level through to brick-red or orange-black at the upper limit of its range. This is probably due to leaching and ferric oxide deposition which is quite common among the Leptonacea and other small bivalves.

ORIENTATION OF ARTHRITICA TO HOST

Experiments with living specimens were carried out at the Zoology Department's Marine Station at Island Bay, Wellington. As neither *Arthritica* nor *Pectinaria* occurs in the vicinity of the Marine Station, the laboratory sea-water supply had not come in contact with either animal prior to the experiments. To eliminate errors arising from possible phototactic responses in *Arthritica* all experiments were carried out in a darkened room.

The laboratory experiments were designed to determine: (1) If, in *A. bifurca*, there was any response to the presence of *Pectinaria australis* when the two species were separated in an aquarium. (2) The nature and degree of this response (if present) and to what extent *Arthritica bifurca* could distinguish between a water current from the host and a control current which had not come in contact with the host species.

A. bifurca moves with a gliding motion similar to that described for *A. crassiformis* by Ponder (1965). However, large numbers of *A. bifurca* in a dish of sea water moved neither rapidly nor at all constantly, and did not show mutual attraction resulting in the formation of clusters of individuals, as shown by the non-commensal *Lasaea rubra* (Eulamellibranchia, Erycinacea) under similar conditions (Morton, 1960).

As the moving polychaetes were found to leave a readily traceable furrow in fine compacted black sand brought from Petone Beach, it was not necessary to use the "talcum powder" technique suggested by Morton (1962).

Experiments were carried out using bivalves ranging from 0.40 mm. to 4.25 mm. in shell length.

Investigation 1 (Fig. 5)

(a) *Response to host stimulus*

A living *Pectinaria* was placed in a 7.5 cm. × 2.5 cm. glass vial filled with sand. With the mobility of the animal thus restricted the vial was sunk in the center of a 46 cm. × 28 cm. clear plastic tank containing about 10 cm. of fine sand and filled with fresh sea water. Two bivalves were placed on the surface of the sand in each of the four corners of the tank, and the experiment was left in a dark room for 12 hours. The water was in no way aerated or renewed.

The furrows left in the sand by moving bivalves were traced for each of five runs. A tracing of the fourth run in this series is shown in Figure 5. On three occasions all eight *Arthritica* located *Pectinaria* and on two occasions all but one of the bivalves were found on the rim of the tube.

(b) *Speed of movement*

In dim red light a similar system was set up, and with the aid of a stop-watch bivalves were timed as they moved towards the host. At two-minute intervals the distance travelled was recorded by measuring the length of the sand furrows with dividers. The distance travelled per second by moving *Arthritica* was calculated for each two-minute period. Bivalves 15 cm. to 20 cm. from *Pectinaria* (measured in a direct line) moved at 0.08 cm. per second, but within 5 cm. of the host this rate was increased to 0.20 cm. per second.

Interpretation

This investigation established that *Arthritica bifurca* is attracted to the exhalant currents of *Pectinaria*. Figure 5 also illustrates that the closer the bivalves get to the worm tube the more direct is the route taken, implying that the attractive force increases with proximity to its source. Close to *Pectinaria* the increased rate of movement shown by *Arthritica* is a direct orthokinetic response. However, there was no evidence of a consistent klinokinetic response. In some cases the rate of change of direction decreased nearer to the host, but in others it did not, and therefore no general pattern could be established.

Investigation 2

Random choice of host (Table I)

A 46 cm. × 28 cm. clear plastic tank containing about 10 cm. of sand was filled with sea water. Fifty bivalves, and five tube worms, ranging between 20 mm. and 70 mm. in tube length, were scattered on the surface of the sand and left for 12 hours in a dark room. The water was not aerated or renewed. This experiment was carried out 10 times using different specimens of *Arthritica* and *Pectinaria* on each occasion. The percentage of bivalves found on the rim of a

Fig. 4

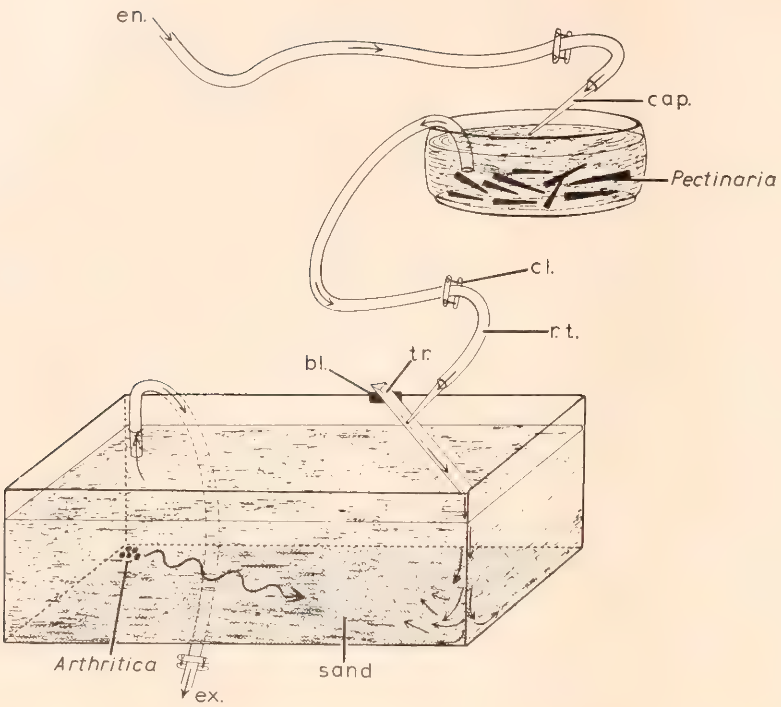
en.
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Fig. 5

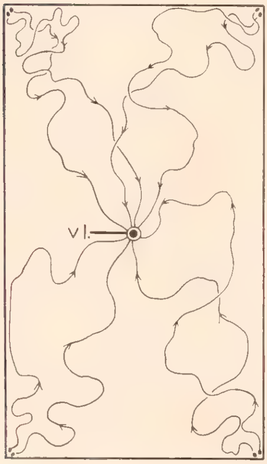

Fig. 6

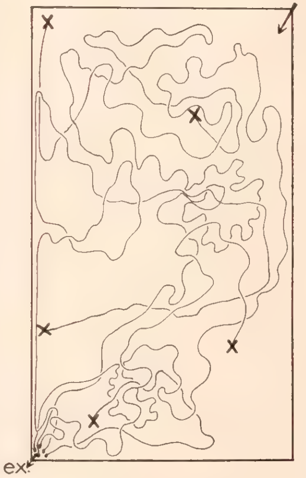

Fig. 7

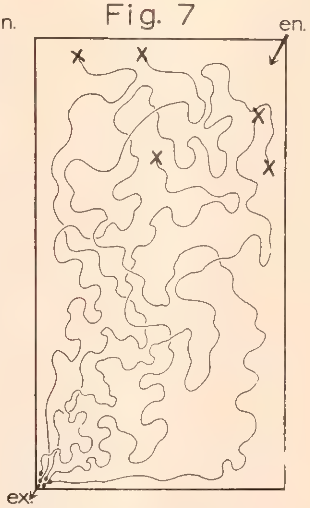

FIGURE 4. Diagram of apparatus used in Investigation 3: bl., wooden block; cap., capillary tube; cl., clamp; en., sea water entry; ex., sea water exit; r. t., rubber tubing; tr., trough. Direction of water flow indicated by arrows. General direction of *Arthritica* migration indicated by waving line.

TABLE I

Investigation 2—Percentage of Arthritica found in association with Pectinaria after 12 hours

Run No.	1	2	3	4	5	6	7	8	9	10
% success	90	92	86	88	96	98	82	86	90	88

Pectinaria tube after 12 hours is shown in Table I. After each run no more than 15 bivalves were found on any one tube.

Interpretation

An average of 90% *Arthritica* located *Pectinaria* in each experiment. This high figure suggests that there is probably some important, but as yet undiscovered, biological reason for its association with *Pectinaria.* Bivalves were found around the rims of all sizes of worm tube in approximately equal numbers, showing that *Arthritica bifurca* has no preference for *Pectinaria* of any specific size.

As *Pectinaria australis* is the only representative of the family Pectinariidae known from New Zealand, it was not possible to run a control experiment to determine host specificity.

Investigation 3

Response to host and control currents (Figs. 4, 6, 7)

The experiment was set up as shown in Figure 4, with balanced inflow and outflow of sea water. Inflowing water passed through a bowl containing 10 *Pectinaria* and then descended down one corner of the tank (en.) and was led out at the diagonally opposite corner (ex.). In this tank five bivalves were placed at the outflow end. The experiment was carried out five times in a dark room, each time with a control experiment having no *Pectinaria* in the inflow bowl. The first three runs were of 12 hours duration, but these resulted in an extremely complex trail pattern. Figures 6 and 7 are tracings of the fifth and final run of both the live and control experiments in which the running time was two hours.

Interpretation

The flow of water over the sand was rather diffuse and this resulted in *Arthritica* taking a less positive direction than might have been the case in a different assembly. Nevertheless there is a definite tendency for *Arthritica* to migrate towards the inflow corner when *Pectinaria* is present in the inflow current (Fig. 7), compared with a rather unoriented trail pattern with no definite evidence of rheotaxis shown

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FIGURE 5. Investigation 1: Tracing of run no. 4. vl., glass vial containing *Pectinaria.*

FIGURE 6. Investigation 3: Tracing of 5th and final control run. Position of *Arthritica* after two hours indicated by "X." en., sea water entry; ex., sea water exit.

FIGURE 7. Investigation 3: Tracing of 5th and final live run. Position of *Arthritica* after two hours indicated by "X." en., sea water entry; ex., sea water exit.

by the control (Fig. 6). The presence of *Pectinaria* therefore causes chemo-orientation of *Arthritica* towards the source of inflowing water.

DISCUSSION

Significant work has been published concerning species of the polynoid genera *Arctonoë, Acholoë, Gattyana, Lepidasthenia, Polynoë, Harmothoë* and *Halosydna,* and the physiology of their commensal relationships with their respective marine invertebrate hosts, by Davenport (1950, 1953a, 1953b) and by Davenport and Hickok (1951). Morton (1962) has shown that *Montacuta ferruginosa* is chemotactically attracted to its heart-urchin host. Conclusions drawn from experimental work in this paper support in principle that marine commensals are capable of finding their way to a specific host by chemotaxis.

Ponder (1965) has discussed biological advantages for *Arthritica crassiformis* in its association with *Anchomasa similis,* but could find no benefits for the pholad. Similarly, I could find no advantages for *Pectinaria australis* in its association with *Arthritica bifurca. Pectinaria* without bivalves attached appeared to be unaffected by their absence. Also, advantages for *Arthritica bifurca* appear to be even more obscure than those suggested by Ponder for *A. crassiformis,* as little protection is provided by the rather temporary worm burrow in the sand. The necessity for a commensal mode of life and the biological reasons for commensalism in the genus *Arthritica* are unknown.

Ponder has pointed out that leptonacean bivalves, by virtue of their small size, efficient mobility, temporary byssus fixation and larval incubation, are particularly adapted to a commensal mode of life with burrowing animals. Ponder has removed shelled prodissoconch larvae from adults of *A. crassiformis* and *A. hulmei* but it is not known at what stage of development larvae are released, or if in fact they are free-swimming on liberation. During March and April large numbers of prodissoconch larvae were found in the specialized brood pouches of *A. bifurca* where they are retained during incubation. However, if larval incubation is carried beyond a pelagic stage in *A. bifurca,* then the ability to locate a host is of no advantage unless the bivalve frequently outlives the worm or becomes separated from it. Larvae have not been observed in the Wellington Harbour plankton, but if there is no short pelagic stage, overcrowding would occur on the *Pectinaria* tube. Therefore, larvae probably leave the parent, and a short planktonic life supported by pelagic or post-pelagic host-finding behavior would be an adequate provision for dispersal of the species.

The experiments illustrated in this paper show that *Arthritica bifurca* is attracted to *Pectinaria australis* chemotactically by way of exhalant water currents from the tube worm, and that there is a direct orthokinetic response as the bivalves move closer to the host.

SUMMARY

1. The bivalve mollusk *Arthritica bifurca* (Webster), previously considered to be free-living, has been found in large numbers living commensally with the tubicolous polychaete *Pectinaria australis* Ehlers.

2. *Arthritica bifurca* is attached by several fine byssus threads to the outer surface of the head-end of the tube of *Pectinaria australis,* orientated vertically with

its anterior end directed downwards. Biological reasons for this commensal relationship are obscure.

3. Ecological observations on the *Pectinaria australis* population at Petone Beach, Wellington Harbour, suggest that the polychaete lives for only 12 or 18 months, and is subject to an extremely high mortality rate during growth and maturation and also after breeding in the spring. There is evidence for a fairly extended breeding period, and the population is probably replenished mainly during late summer by settlement of larvae at or near the high tide level.

4. Larvae of *Arthritica bifurca* are incubated by the parent at least to the shelled prodissoconch stage. A short planktonic larval life supported by pelagic or post-pelagic host-finding behavior is the probable means by which the commensal bivalve is dispersed.

5. Physiological experiments with live material established that *A. bifurca* is able to distinguish between sea water that has passed over the host and sea water which has not done so. The bivalves are attracted chemotactically to *Pectinaria australis* by way of exhalant water currents from the tube worm, and there is a direct orthokinetic response as the bivalves move closer to the host.

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THE INFLUENCE OF PUPAL AGE ON SENSITIVITY TO RADIATION[1]

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In recent years there has been increasing interest in the application of ionizing radiations in the control of insects, and increasing recognition of the usefulness of insects as experimental subjects in the investigation of radiobiological phenomena. For some types of radiobiological study, the germicidal wave-lengths of ultraviolet (UV) radiation, since they are preferentially absorbed by nucleic acids, may be employed more advantageously than the ionizing wave-lengths of x- and gamma radiations. Furthermore, sources of UV radiation are inexpensive, relatively non-hazardous, and readily available. Unfortunately, interpretation of the effects of UV on metazoa may be complicated by the fact that the radiation penetrates only a few cell layers; this explains the paucity of data on UV effects on insects, except for those on insect eggs.

As part of a more extensive investigation of recovery mechanisms in irradiated *Tribolium* (Ducoff and Walburg, 1960; Ducoff and Bosma, 1963) we have compared the effects of x-rays and germicidal UV on pupal stages of *T. confusum.* The two types of radiation differed markedly in regard to stage of greatest sensitivity and to the relative importance of lethal effects and induction of developmental abnormalities.

MATERIALS AND METHODS

The *T. confusum* stock employed was derived from the colony of G. Fraenkel; the beetles were maintained in white flour supplemented (4%) with brewer's yeast, at 30° C. and 70% R. H. Individual cultures were kept in previously washed polystyrene vials fitted with perforated snap-caps. X-irradiation was performed with a Picker Vanguard deep-therapy machine, operated at 280 kvp and 20 ma; HVL was 1.0 mm. copper, and subject-target distance was 40 cm., yielding a dose rate of approximately 250 r/min., as measured with a Victoreen condenser meter. The UV source was a 15-watt germicidal lamp in a desk-type fluorescent fixture; this gave intensities of about 250 ergs/mm.^{2}/min. at a distance of 100 cm. The output of this type of lamp declines with use, so actual dose rate was measured at the end of each exposure series, using a General Electric Ultraviolet Intensity meter. Care was taken to avoid photoreactivation.

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Homogeneous populations of pupae of known age were obtained in the following manner: Young adult males and females in approximately equal numbers were placed in a large vial of fresh medium for a week. The adults were then removed, and the culture incubated undisturbed for an additional two weeks. The culture was then sifted daily, and those animals which had pupated in the preceding 24 hours removed, sexed, and placed in separate vials of fresh medium.

For irradiation, pupae were freed of medium and distributed in plastic petri dishes, which were uncovered during the exposures.

RESULTS

Preliminary experiments were performed with insects exposed to UV two to four days after pupation. Even doses in excess of 12,000 ergs/mm.[2] did not

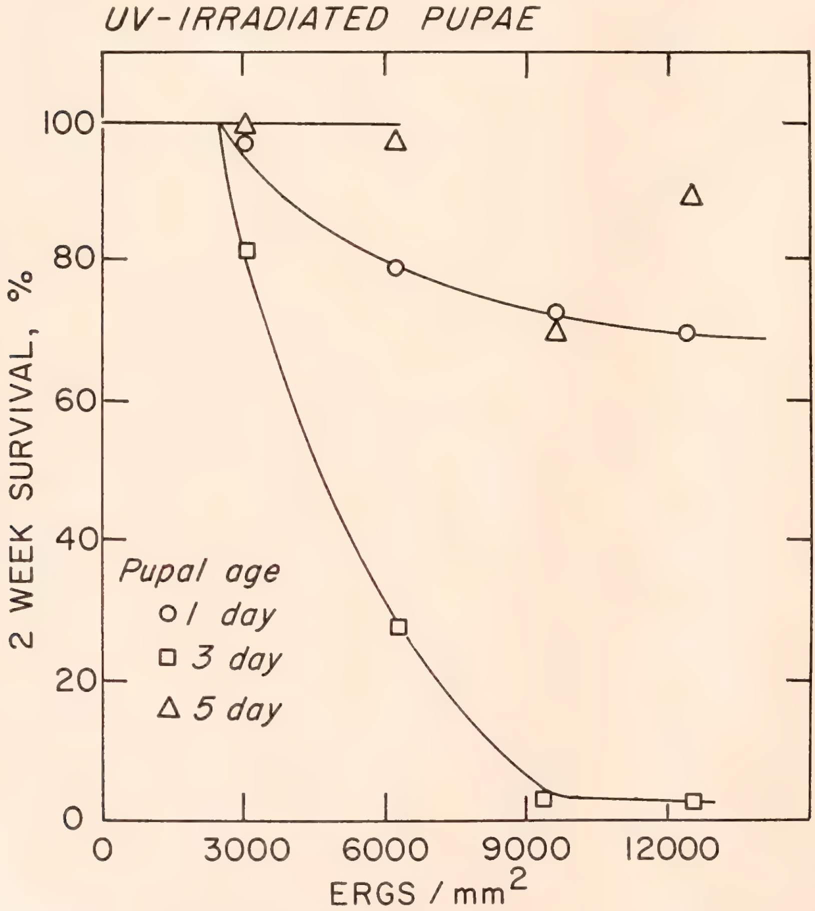

Figure 1. The per cent of *T. confusum* surviving two weeks after exposure, as pupae, to germicidal ultraviolet.

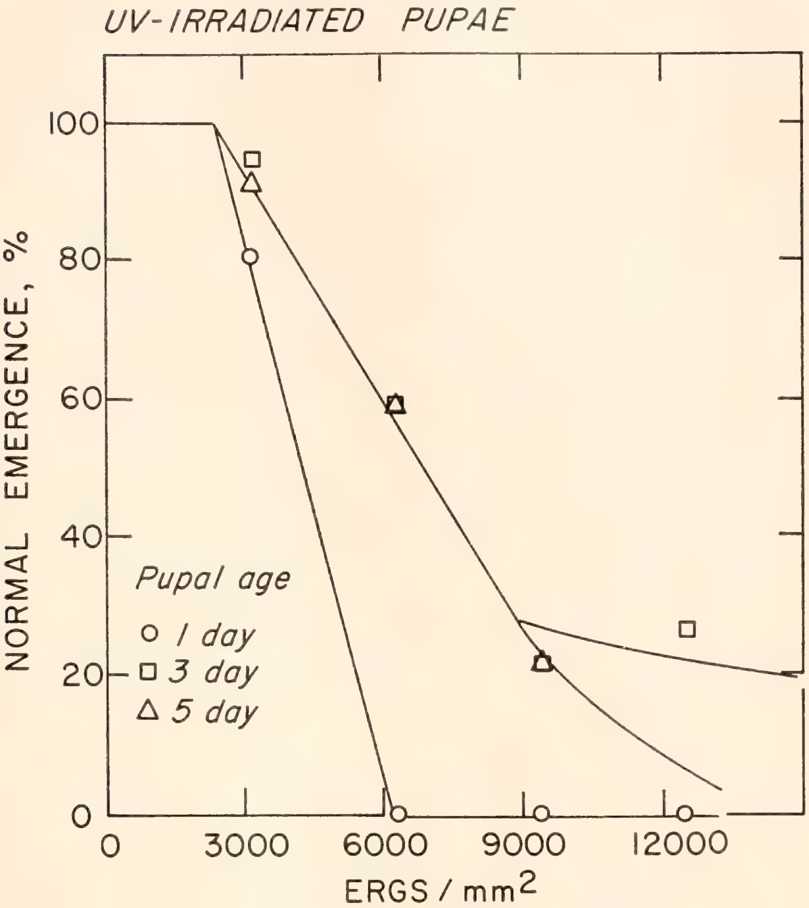

FIGURE 2. The per cent of UV-irradiated *T. confusum* pupae which developed into adults that appeared normal to the unaided eye.

suppress eclosion, but all of the adults exhibited severe wing deformities, and most died during the following week.

When pupae of selected ages were UV-irradiated, there were marked differences in sensitivity. UV radiation was much more effective when administered on the third day of pupation than when administered on either the first or the fifth day, when the parameter scored was survival for two or more weeks after exposure (Fig. 1). On the other hand, if the incidence of emergence without obvious deformities was considered (Fig. 2), the youngest pupae were the most sensitive. Surprisingly, although the abnormalities produced by UV-treatment of one-day and of five-day pupae in these and in similar experiments often appeared severe, they were not necessarily incompatible with life; many adults with gross abnormalities, primarily of the elytra, survived for months after eclosion. By contrast, a number of those UV-irradiated on the third day of pupation gave rise to adults which appeared grossly normal, but which died within a few days.

The sensitivity of three-day-old pupae to the lethal effects of UV was also surprising, in view of reports that *Tribolium* (Beck, 1963; Ducoff and Bosma, 1963) as well as many other insects—*e.g., Drosophila* (Villee, 1946)—show a progressive decline in x-ray sensitivity as pupal development proceeds. Accordingly, a more extensive experiment was performed. Newly-pupated beetles were collected on each of 5 successive days; on the fifth day, sub-groups were exposed to appropriate doses of x- or UV-radiation. The results of the x-irradiation are presented in Table I and those of the UV-irradiation in Table II. These data confirm the previous reports that x-ray resistance increases as pupae develop, with a very abrupt increase about the second day; furthermore, resistance to the production of deformities by x-irradiation parallels resistance to lethal effects. The data also demonstrate that resistance to UV-lethality decreases to a minimum on the third day, and that doses which produce negligible lethality in older pupae nevertheless produce a very high incidence of developmental abnormalities.

Table I

X-irradiated pupae

Pupal age (days)	% Survival, 11 days		% Deformed	
	3 kr	5 kr	3 kr	5 kr
1	95	20	95	100
2	75	75	25	80
	9 kr	13 kr	9 kr	13 kr
3	75	65	50	43
4	95	90	9	10
5	100	100	0	0

Table II

UV-irradiated pupae

Pupal age (days)	% Survival, 11 days		% Deformed among survivors	
	4200 ergs/mm.^{2}	6500 ergs/mm.^{2}	4200 ergs/mm.^{2}	6500 ergs/mm.^{2}
1	85	55	90	90
2	80	40	100	100
3	30	5	80	100
4	89	80	20	80
5	90	90	30	80

Discussion

Sub-lethal doses of UV-radiation proved to be much more efficient in producing developmental abnormalities than sub-lethal or mid-lethal doses of x-radiation.

This result was not unexpected. It seems likely that damage to deeper tissues is necessary to cause death, and that very high doses of the poorly penetrating UV wave-lengths may produce extensive damage to surface structures without producing significant effects in the deeper tissues. In accordance with this, the x-ray effect is highly specific, producing deformities only in the elytra and in the posterior wings (Beck, 1963); thus, these deformities appear to be phenocopies of the mutant "split" (McDonald, private communication). Most of the readily-observable UV-induced deformities also involved the wings, but the wing deformities after UV-irradiation were much more varied both in type and in severity, and deformities in other parts of the body were not infrequent.

The spectacular increase in UV-sensitivity on the third day of pupation is more difficult to explain. There may be particularly sensitive cellular processes occurring, or morphogenetic movements may bring sensitive structures close to the surface at that time. Alternatively, the concentration of nucleotides or other UV-absorbing materials might be reduced on the third day. The data of Chaudhary and Lemonde (1963) and of Devi *et al.* (1963) constitute evidence against this possibility, however; these workers found a slightly lowered nucleotide concentration on the first or second day of pupation, and a marked and progressive increase of 260 mμ-absorbing material from prepupal to early adult stages.

The time of maximum sensitivity of *Drosophila* pupae also was different for x-rays (Villee, 1946) than for UV-light (Villee, 1947), and for lethality as compared to phenocopy production by UV. In particular, the flies were more sensitive to killing and more resistant to phenocopy-induction an hour or two after pupation than at any other time during pupal or late larval development. Unfortunately, Villee's interesting UV experiments were performed prior to the recognition of the phenomenon of photoreactivation (Kelner, 1949) and presumably, no precautions were taken to avoid light exposure. Subsequently, Perlitsh and Kelner (1953) demonstrated photoreactivation of phenocopy-production after UV-irradiation of newly-pupated *D. melanogaster.* Thus it is not clear whether the changes in apparent UV-sensitivity in Villee's experiments truly represent changes in sensitivity, or reflect changes in photoreactivating efficiency. It is clear, however, that in *Drosophila,* as in *Tribolium,* the time of maximum sensitivity to the lethal effect of UV does not coincide with the time of maximum sensitivity to UV-induction of developmental abnormalities.

SUMMARY

T. confusum exposed to germicidal UV at any time during the 5-day pupal period exhibits a high incidence of gross morphological deformities, particularly of the wings, in the emerging adult, although the greatest sensitivity to this effect occurs on the first day of pupation. Mortality within two weeks is by far the greatest, however, if UV-irradiation is performed on the third day of pupation. By contrast, sensitivity to both lethality and abnormality-induction after x-ray exposure is greatest on the first day of pupation and declines progressively thereafter, with the sharpest decline occurring late on the second day. These differences are partially explained by the fact that energy absorbed from x-rays is distributed rather uniformly throughout the pupa, whereas most of the UV energy is absorbed in the outermost tissue layers.

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EFFECTS OF DIMETHYLSULFOXIDE ON PRODUCTIVITY OF X-IRRADIATED FLOUR BEETLES[1]

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Chemical and physical agents are known to modify the degree of injury to biological systems subjected to radiation. Some of these agents by themselves might induce genetic damage (chromosomal aberrations) which may lead to illness or death of the organism. Successful eradication of the screw worm fly from Curaçao Island and the southeastern United States, by releasing males sterilized by gamma radiation (Baumhover *et al.,* 1955), has stimulated parallel research using chemical sterilants. The literature concerns predominately applied aspects for insect control (see reviews: Weidhaas and McDuffie, 1963; Smith, 1963; and Smith *et al.,* 1964).

A chemosterilant effect (inhibited ovarian growth) was reported for folic acid antagonists in *Drosophila* (Goldsmith and Frank, 1952), for various mitotic poisons and tumor-inhibiting agents in house flies (Mitlin and Baroody, 1958), and for alkylating agents in house flies (LaBrecque, 1961), screw worm flies (Crystal and LaChance, 1963), and mice (Moutschen, 1961). Detailed studies of the chemosterilant effect on insect oogenesis were found for fruit flies (Cantwell and Henneberry, 1963), house flies (Morgan and LaBrecque, 1962) and mosquitos (Rai, 1964). Effort is expanding to study the biological effects of chemosterilants and the mechanisms involved which result in modified insect productivity.

On the other hand, chemicals are sought for their radioprotective abilities. Ashwood-Smith (1961a) reported a 70% radioprotection of dimethylsulfoxide (DMSO) for whole-body acute x-ray exposures to mice.

The present paper reports that DMSO showed no radioprotective properties on oogenesis in flour beetles but had an indirect chemosterilant influence on flour beetle productivity. Microscopic examination of ovarioles showed that oogonial differentiation and growth were adversely affected, due to starvation resulting from unpalatability of food containing DMSO.

MATERIALS AND METHODS

General

In these experiments, sexually-mature (three-week-old), virgin female flour beetles, *Tribolium castaneum* (Herbst), mutant: sooty, from the laboratory of Dr. A. Sokoloff, Berkeley, California, were treated with DMSO. After x-irradiation, females were mated to control males of the same age. X-irradiation was performed

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at 250 kvp. 30 ma, H.V.L. 0.86 mm. copper, 0.25 mm. copper + 1.0 mm. aluminum filtration and an anode-subject distance of 2.5 inches, and 1 kr/min. was delivered, as measured in air by a Victoreen Dosimeter. The flour beetles were maintained in conventional food (5% brewer's yeast, 95% whole-wheat flour) and at 32 ± 1° C. and 65–70% relative humidity. Dimethylsulfoxide, considered to have a density of 1, was diluted in distilled water.

Fecundity (mean number of eggs/♀/day), fertility (mean number of adult F_1/♀/day), and viability (total number of adult F_1/total number of eggs) were the productivity parameters measured. Dissections of ovarioles and whole-mount photomicrographs present the details of oogenesis.

Experiment 1

Sooty females were placed for 5 minutes on filter paper wetted with 0, 1, 10, and 100% DMSO, then x-rayed ½ hour after DMSO treatment with 0, 1, 2, and 4 kr. Wetted filter paper was used in order to increase the time of exposure and to eliminate drowning which occurred when beetles were dipped. Triplicate samples of five pairs in 10 g. of food were made and fresh food was given three times weekly during a 12-day period. At these times, eggs were counted and allowed to develop into adults, which were counted one month later.

Experiment 2

Sooty females were dipped three minutes in 67% DMSO. Three hours later they were exposed to 0, 2, and 4 kr. Subsequently, productivity measurements similar to those in Experiment 1 were made on five single pairs per treatment for 14 days.

Experiment 3

Sooty females were placed for 10 minutes on filter paper wetted with 67% DMSO. After 15 minutes and after 20 hours, they were x-irradiated with 0, 1, 2, and 4 kr. Three replicates of 10 pairs per 10 g. food were observed for 18 days under conditions similar to the other experiments, except that only the number of adult F_1 was counted.

Experiment 4

Sooty females were placed for four days on food containing 0, 4.6, and 9.1% DMSO, after which they were x-rayed with 0, 2, and 4 kr, and then mated. Five replicates of single pair-matings of these beetles were established on food containing no DMSO and thereafter lethality and productivity as described in Experiment 1 were studied.

Experiment 5

This experiment was repeated and the results were comparable; therefore, the data are treated as one experimnt. Sooty females were kept for 0, 1, 4, and 11 days on food containing 0, 4.6, and 9.1% DMSO or no food. On these days, females were preserved in 70% alcohol until dissection in insect Ringer solution. More than 20 whole-mount dissections were made and representative examples were photomicrographed.

RESULTS

Productivity Data

Experiment 1

Fecundity, fertility, and viability progressively decreased with 2- and 4-kr x-ray exposures to approximately the same extent regardless of DMSO treatment (Table

TABLE I

PRODUCTIVITY MEASUREMENTS
Sexually Mature (3-week old) Virgin Female Flour Beetles, *Tribolium castaneum* (Herbst), mutant: sooty, X-Rayed One-Half Hour After 5 Minutes on Filter Paper Wetted with Dimethylsulfoxide (DMSO)

	X-Ray in kR			
% DMSO	0	1	2	4
	FECUNDITY (mean numbers of eggs/ ♀ /day)			
0	15 ± 0.4*	14 ± 0.7	13 ± 0.2	8 ± 0.5
1	13 ± 0.2	11 ± 0.4	11 ± 0.6	7 ± 0.5
10	14 ± 0.3	13 ± 1.4	11 ± 0.2	7 ± 0.5
100	All Died			
	FERTILITY (mean numbers of adult F_1/ ♀ /day)			
0	12 ± 0.4*	10 ± 0.9	6 ± 0.4	0.6 ± 0.005
1	11 ± 0.7	8 ± 0.8	4 ± 0.5	0.5 ± 0.1
10	12 ± 0.1	10 ± 1.0	5 ± 0.2	0.7 ± 0.1
	VIABILITY (fertility/fecundity)			
0	0.80 ± 0.03*	0.71 ± 0.05	0.46 ± 0.02	0.08 ± 0.01
1	0.85 ± 0.03	0.72 ± 0.03	0.36 ± 0.02	0.07 ± 0.03
10	0.86 ± 0.01	0.77 ± 0.01	0.46 ± 0.02	0.10 ± 0.03

*Standard Error

I). These parameters of productivity were not modified by DMSO for a given x-ray exposure. A 1-kr exposure resulted in responses comparable to control values.

X-radiation was highly significant in altering each productivity datum (Table II); DMSO was a statistically significant factor because of low productivity values at 1% DMSO. Values at 0 and 10% DMSO were comparable; therefore, differences among values within a given x-ray exposure were considered as biological variability. No significant effects could be attributed to an interaction between x-ray and DMSO.

Table II

ANALYSIS OF VARIANCE
Square Root Transformation of Productivity Data from Sexually Mature (3-week old) Virgin Female Flour Beetles, *Tribolium castaneum* (Herbst), Mutant: Sooty, Placed for 5 Minutes on Filter Paper Wetted with Dimethylsulfoxide (DMSO) and X-Rayed One-Half Hour Later.

Error Source	Degrees of Freedom	Sum of Squares	Mean Square	F Value
Fecundity				
1-DMSO	2	0.44	0.22	9.35 *
2-X-Ray	3	5.43	1.81	77.55 *
1-2 Interaction	6	0.07	0.01	0.49
Fertility				
1-DMSO	2	0.59	0.29	10.82 *
2-X-Ray	3	37.95	12.65	464.76 *
1-2 Interaction	6	0.17	0.03	1.07
Viability				
1-DMSO	2	0.05	0.03	6.96 *
2-X-Ray	3	4.52	1.50	398.17 *
1-2 Interaction	6	0.02	0.003	0.86

*Significant at the 5% Level

Experiment 2

A. Fecundity of females (Table III)

1. Not treated with DMSO: Comparable numbers of eggs were oviposited at 0- and 2-kr x-ray exposures.
2. Treated with DMSO: At 2- and 4-kr x-ray exposures, comparable numbers of eggs were oviposited which were significantly fewer compared to 0-kr values. Perhaps the dehydrant effect of DMSO impaired females in activity and/or oviposition.

TABLE III

PRODUCTIVITY MEASUREMENTS
Sexually Mature (3-week old) Virgin Female Flour Beetles,
Tribolium castaneum (Herbst), Mutant: Sooty, were X-Rayed
3 Hours After a 3 Minute Dip in 67 Percent Dimethylsulfoxide (DMSO)

	X-Ray in kR		
% DMSO	0	2	4
	FECUNDITY (mean number of eggs/ ♀ /day)		
0	17 ± 0.6*	18 ± 1.2	11 ± 2.3
67	21 ± 0.7	12 ± 1.5	11 ± 0.8
	FERTILITY (mean number of adult F_1/ ♀ /day)		
0	14 ± 0.9	9 ± 0.8	1 ± 0.1
67	15 ± 2.6	5 ± 1.6	1 ± 0.2
	VIABILITY (fertility/fecundity)		
0	0.82 ± 0.02	0.50 ± 0.03	0.09 ± 0.04
67	0.71 ± 0.1	0.41 ± 0.1	0.09 ± 0.03

*Standard Error

B. Fertility (Table III)

Fertility of untreated and DMSO-treated females was progressively reduced to the same extent by increasing x-ray exposures but was not altered for a given x-ray exposure.

C. Viability (Table III)

For untreated or DMSO-treated females the numbers of eggs which developed into adults progressively decreased with greater x-ray exposures.

D. Analysis of Variance (Table IV)

1. X-irradiation and not DMSO was the effective agent in modifying the productivity responses of female flour beetles.
2. The F-value, 6.04, for the interaction between DMSO and x-rays was significant only for fecundity. This might be explained by the low mean number of eggs of DMSO-treated females given 2 kr.

TABLE IV

ANALYSIS OF VARIANCE
Square Root Transformation of Productivity Data from Sexually Mature (3-week old) Virgin Female Flour Beetles, Tribolium castaneum (Herbst), Mutant: Sooty, X-Rayed 3 Hours After a 3 Minute Dip in 67 Percent Dimethylsulfoxide (DMSO)

Error Source	Degrees of Freedom	Sum of Squares	Mean Squares	F Value
Fecundity				
1-DMSO	1	0.03	0.03	0.16
2-X-Ray	2	6.51	3.26	16.39 *
1-2 Interaction	2	2.40	1.20	6.04 *
Fertility				
1-DMSO	1	0.67	0.70	1.75
2-X-Ray	2	36.13	18.07	47.17 *
1-2 Interaction	2	1.33	0.67	1.74
Viability				
1-DMSO	1	0.08	0.08	2.33
2-X-Ray	2	2.94	1.47	40.93 *
1-2 Interaction	2	0.03	0.02	0.48

*Significant at the 5% Level

Table V

MEAN NUMBERS OF F_1 ADULTS PER FEMALE PER DAY WHEN SEXUALLY MATURE (3-WEEK OLD) VIRGIN FEMALE FLOUR BEETLES, Tribolium castaneum (HERBST), MUTANT: SOOTY, WERE PLACED FOR 10 MINUTES ON FILTER PAPER WETTED WITH DIMETHYLSULFOXIDE (DMSO) AND THEN X-RAYED 15 MINUTES OR 20 HOURS LATER.

	X-Ray in Kr			
% DMSO	0	1	2	4
X-Rayed After 15 Minutes				
0	14 ± 1.3*	11 ± 0.9	7 ± 0.5	1 ± 0.3
67	12 ± 1.1	11 ± 1.2	6 ± 0.7	1 ± 0.2
X-Rayed After 20 Hours				
0	13 ± 1.4	11 ± 0.9	7 ± 0.6	1 ± 0.2
67	11 ± 0.8	11 ± 0.9	7 ± 0.6	1 ± 0.3

* Standard Error

At 4 kr the productivity measurements for untreated and DMSO-treated females were significantly and comparably reduced compared to those of other radiation exposures, indicating radiation induced irreparable damage to developing mature oocytes. Likewise, the 2-kr exposure reduced fertility and viability significantly from control values.

Experiment 3

A. Fertility (Table V)
   1. Was the same for 0 and 1 kr but was progressively reduced by 2 and 4 kr.
   2. Was comparable for untreated and DMSO-treated females given a specified x-ray exposure.
   3. Was not changed whether x-ray was given 15 minutes or 20 hours after DMSO treatment.

Experiment 4

In addition to results previously stated concerning x-ray effects on productivity, data of this experiment (Table VI) showed that flour beetle females subjected

TABLE VI

PRODUCTIVITY DATA SUMMARY FOR SEXUALLY MATURE (3-WEEK OLD) VIRGIN FEMALE FLOUR BEETLES, *Tribolium castaneum* (HERBST), MUTANT: SOOTY, FED FOUR DAYS ON FOOD CONTAINING DIMETHYLSULFOXIDE (DMSO)

% DMSO	Kr X-Ray	1	2	3	2	5	3	Percent Parental Lethality	Percent Parental Fertility
		Number of Days in Productivity Period							
0	0	5/5*	5/5	5/5	5/5	5/5	5/5	0	100
		5 **	15	16	17	18	17		
4.6	0	0/5	5/5	5/5	5/5	5/5	5/5	0	100
		0	8	13	15	12	13		
9.1	0	0/5	0/5	1/3	2/3	2/3	2/3	40	40
		0	0	21	17	15	18		
0	2	5/5	5/5	5/5	5/5	5/5	5/5	0	100
		5	8	5	6	6	7		
4.6	2	0/5	3/5	3/5	3/5	3/5	3/5	0	60
		0	2	8	10	6	6		
9.1	2	0/5	0/5	1/2	1/2	1/2	1/2	60	20
		0	0	5	4	5	4		
0	4	3/5	5/5	5/5	2/5	2/5	0/5	0	100
		1	3	0.7	0.2	0.1	0		
4.6	4	0/5	1/5	2/4	0/4	2/4	0/4	20	40
		0	2	2	0	0.4	0		
9.1	4	0/5	0/5	0/3	0/3	0/3	0/3	40	0
		0	0	0	0	0	0		

* Number Reproducing ♀♀/Number ♀♀Tested

** $\bar{X}$ Number of Adult F_1/ ♀ /day.

to food containing DMSO for four days and then x-rayed had: (1) increased lethality at 9.1% DMSO; (2) progressively decreased fertility; (3) delayed onset of productivity by one day at 4.6% DMSO and at least three days at 9.1% DMSO. Delay of reproductive onset was found also when no x-ray treatment was given; therefore, this response was not due to x-radiation but to DMSO-contaminated food resulting in failure to feed and oosorption. These females were not observed cytologically since subsequent production of F_1 progeny was indicative of functioning ovarioles. Females on 9.1% DMSO then given 4 kr never reproduced because the radio-resistant mature oocytes were resorbed, due to the unpalatibility of DMSO on food, and the radiosensitive immature oocytes were irreparably damaged by this quantity of x-ray.

Ovariole Cytology

Experiment 5

Representative ovarioles of flour beetles sacrificed after 0, 1, 4, and 11 days on control food (no DMSO) are shown in Figure 1. In all dissections, a female had paired ovaries, each one of which consisted of 6 ovarioles. Progressing distally, one finds large (mature) oocytes ready for oviposition, oocytes in different stages of growth, differentiating oocytes, the gametorium, and the terminal filament. Similar ovarioles were observed in females cultured for one day on food containing 4.6% or 9.1% DMSO or kept without food. Thus, regardless of treatment during this time, no differences appeared in the size or number of ovarioles or oocytes.

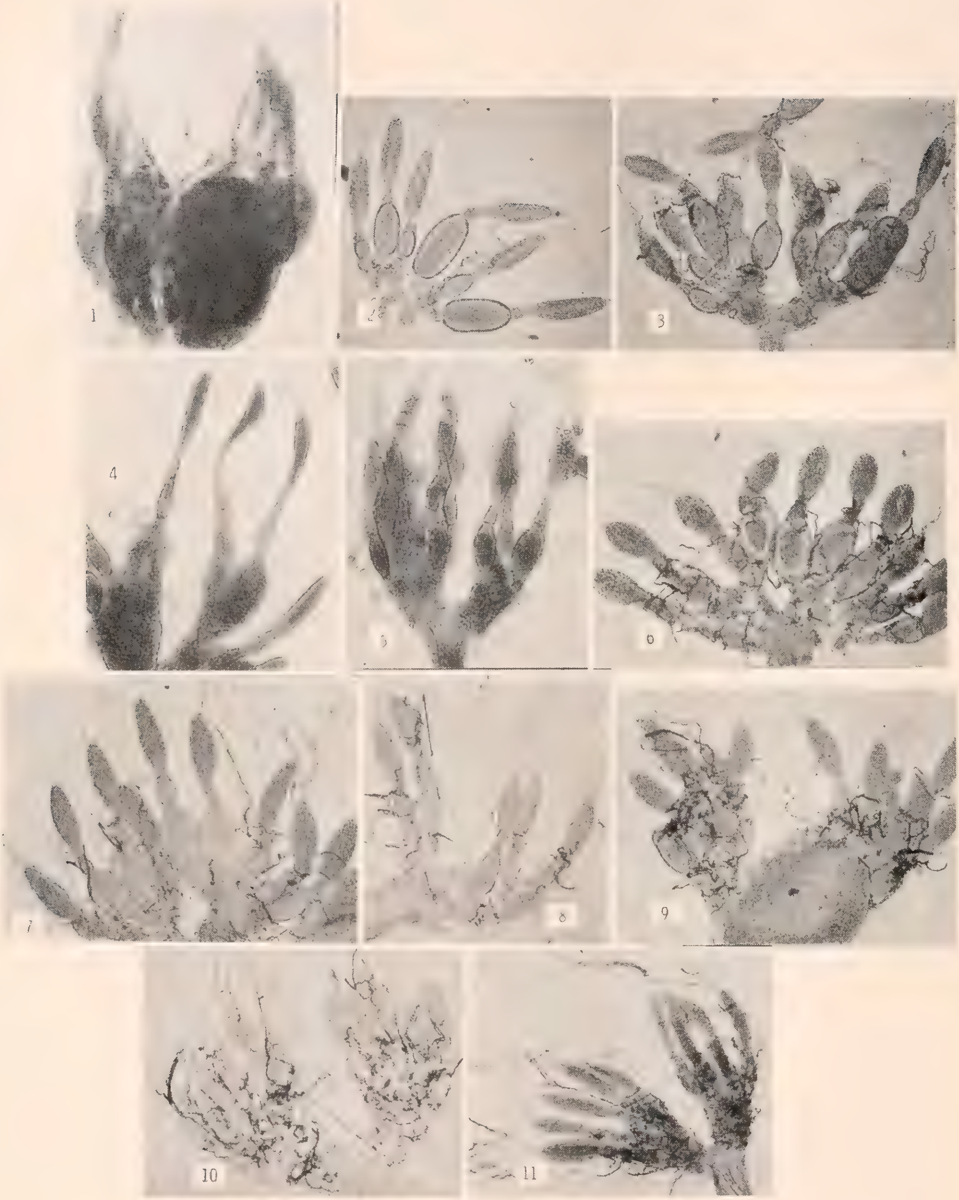
As expected from the productivity data in Experiment 4, ovariole deterioration was observed in females which spent four days on food containing 4.6% DMSO (Figs. 4, 5). In a few females, large oocytes were observed which could, perhaps, have been oviposited. When oocytes no longer differentiated (after four days), the ovarioles appeared thin, shortened, and empty (Figs. 8, 9, 11). Comparable observations were found in females kept four days on 9.1% DMSO in food (not shown). No oocyte appeared mature enough for oviposition and ovariole degeneration was pronounced. Since some (two out of five) females produced a few F_1 (total of 24 per reproducing female during 15 days), some germ cells survived (Table VI).

Compression of the beetle abdomens indicated starvation, which resulted in sterility due to oosorption. Ovarioles from beetles kept without food and dissected on days 3, 5, and 11 (Figs. 2, 3, 6, 7, 10) showed no differences from those of beetles kept on food containing DMSO (Figs. 4, 5, 8, 9, 11). Thus females of *Tribolium* kept without food or unpalatable food absorb mature eggs and fail to mature others.

DISCUSSION

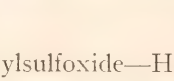
Topical application of DMSO gave no protection to female flour beetle germ cells subjected to x-irradiation. The high (70%) radiation-protective ability of DMSO in mice exposed to whole-body acute lethal x-radiation (Ashwood-Smith, 1961a) was not found to decrease x-ray damage in mice testes, even though it was considerably concentrated in the testes (Ashwood-Smith, 1961b). An attempt to subject female flour beetles to low concentrations, 4.6% and 9.1%, of DMSO over a long period of time (days *vs.* minutes) showed that productivity was reduced after four days. Three out of five females given 9.1% DMSO in their food but not x-rayed never regained their reproductive ability. Of the two productive females, one began reproduction during days 4–6 and the other during days 7–8. Two of the three females not producing died during the first three days. Decreased fecundity might result from chemosterilant-induced interference with the progress of normal oogenesis.

Some biological alkylating agents, such as tepa (Borkovec, 1962), are the most effective male insect chemosterilants—eggs laid by females mated to treated males failed to hatch. These agents were used to sterilize both sexes of species of mosquitos, *Aedes* (Weidhaas *et al.*, 1961; Bertram, 1963; Rai, 1964) and *Culex* (Murray, 1963), females of house flies (LaBrecque, 1961; Morgan and LaBrecque, 1962), of screw worm flies (Chamberlain, 1962) and of *Drosophila* (Cantwell and Henneberry, 1963). Widespread use of these chemicals is restricted because of



FIGURES 1–11.

Whole-mount photomicrographs (82 × magnification) of representative ovarioles of sexually mature flour beetles, *Tribolium castaneum* (Herbst), mutant: Sooty, cultured at 32° C. and 65–70% relative humidity. Figure 1, from control flour beetles on days 1 and 11; Figures 2 and 3, from flour beetles kept three days without food; Figures 4 and 5, from flour beetles kept four days on food containing 4.6% DMSO; Figures 6 and 7, from flour beetles kept without food for 5 days; Figures 8 and 9, from flour beetles kept 8 days on food containing 4.6% DMSO; Figure 10, from flour beetles kept 11 days without food; Figure 11, from flour beetles kept 11 days on food containing 4.6% DMSO.

their possible mutagenic and carcinogenic properties (Auerbach, 1958). Chang *et al.* (1964) reported effective male house fly chemosterilants, hexamethylphosphoramide and hexamethylmelamine, which are non-alkylating agents and have low mammalian toxicity. Compared to these chemicals, the unrelated and simple molecular structure of dimethylsulfoxide— $H_3C—\overset{\overset{O}{\uparrow}}{S}—CH_3$—was not expected to affect gametogenesis adversely.

Critical examination of the females given food containing DMSO revealed the contracted appearance of their abdomens and suggested that starvation resulting in oosorption caused the decreased productivity, rather than DMSO *per se.* Observations of ovarioles from females kept without food were the same as those from females subjected to DMSO-food. Ovarioles from both treatments progressively deteriorated with time.

Microscopic examinations indicated that flour beetle ovarioles were panoistic; however, to be conclusive this will be studied in more detail by incorporating cytochemical techniques. Panoistic ovarioles are distinguished from meroistic ovarioles in that the former have no trophocytes. Types of meroistic ovarioles are: (1) telotrophic, having trophocytes in the germarium; and (2) polytrophic, having syncytia each consisting of trophocytes and an egg cell enclosed in cellular follicles. In the apparent absence of nutritive cords (Bryan, 1954), a detailed study of oogenesis is required to determine whether the apical cells in the germarium are oocytes or trophocytes. Stein (1847), Gross (1903), and Imms (1948) reported polytrophic ovarioles for the coleopteran suborder *Adephaga* and telotrophic ovarioles for the rest of the beetles. On the other hand, Weber (1933) and Wigglesworth (1950) reported telotrophic ovarioles for the *Adephaga* and polytrophic ovarioles for the *Polyphaga.* Bonhag (1958) concluded the existence of panoistic ovarioles in the Coleoptera was not established.

Concentrations as low as 4.6% DMSO indirectly caused sterility by making the food unpalatable and by the subsequent utilization of oocytes as an energy source. Preliminary observations of approximately 100 adult beetles from stock cultures to determine the concentration of DMSO allowing propagation after 5 weeks showed that no F_1 were obtained on 2.3% DMSO food, three F_1 were obtained on 1.8% and 1.4% DMSO food and more than 100 F_1 were produced on 0.9% DMSO food. The 2.3% DMSO food induced 95% lethality in adults after three weeks. Due to these findings, future work is planned to test the usefulness of DMSO as an insecticide or insect repellant on stored products such as foods, clothes, and tobacco.

I am grateful to Mr. R. J. Herschler of the Crown Zellerbach Corporation at Camas, Washington for supplying the DMSO; to Mr. R. L. Buschbom for programming the statistical analysis, and to Mrs. E. H. Jaschek for assisting in the laboratory.

SUMMARY

Sexually mature virgin female flour beetles, *Tribolium castaneum* (Herbst), mutant: sooty, were treated with dimethylsulfoxide (DMSO) and x-rayed. Fecun-

dity, fertility and viability were measured. DMSO gave no radiation protection to female germ cells. Food containing 4.6% DMSO was unpalatable. Photomicrographs of ovarioles showed that sterility resulted because of oosorption. DMSO might be economically important in protecting stored products from insects.

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FACTORS AFFECTING ACTIVITY AND BURROWING HABITS OF THE PINK SHRIMP, PENAEUS DUORARUM BURKENROAD

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The purpose of this study was to determine the effects of light, temperature, salinity, fatigue, persistent daily rhythms, availability of food, bottom types, and bottom water currents on the daily activity and burrowing habits of adult pink shrimp (*Penaeus duorarum*), and to attempt an estimate of their relative importance. Results of modified *in situ* and controlled observations were combined to present a better assessment of behavior in the natural habitat.

Some of the factors influencing the activity and burrowing habits of certain penaeid shrimp have been investigated directly or indirectly in several previous studies. Williams (1958) examined the relation between substrate and burrowing; Eldred (1958) noted the effects of colored light and molting; Williams (1955) and Eldred *et al.* (1961) suggested the effects of low temperature; Wheeler (1937) and Racek (1959) attempted to elucidate the influence of lunar periodicity on activity; Egusa and Yamamoto (1961) demonstrated the effects of low oxygen tension; Dall (1958), Ghidalia and Bourgois (1961), Aaron and Wisby (1964), and Fuss (1964) referred to the possible influence of moon phase or light level. The lack of agreement among some of the above works, particularly concerning the effects of light and lunar periodicity, prompted this investigation.

It is now well established that pink shrimp do burrow into the substrate and are usually active at night (Eldred *et al.*, 1961; Fuss, 1964). Methods of burrowing have been described by Dall (1958) for *Metapenaeus mastersii;* by Egusa and Yamamoto (1961) for *Penaeus japonicus;* and by Fuss (1964) for *P. duorarum.* All of the reported burrowing methods are generally similar.

The observations and experiments reported here are part of a general study by the U. S. Fish and Wildlife Service, Bureau of Commercial Fisheries Gear Research Station in Panama City, Fla., to learn the basic behavior of adult penaeid shrimp.

MATERIALS AND METHODS

In the initial phases of this study the modified *in situ* approach was chosen to eliminate the effects of habitat modification. We first made gross observations on the methods of burrowing, the degree of bottom penetration, and general diurnal cycles of activity (Fuss, 1964), none of which required controlled experiments. As the study progressed we decided to incorporate a series of controlled and partially controlled aquarium observations to supplement field data and provide a quantitative measure of the effects of certain factors on the behavior of *P. duorarum.*

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The limitations of data obtained from aquarium studies in the interpretation of gross behavior have been emphasized by a number of workers (Walford, 1958; Kavanau, 1964; and others), and are presented here to assist in the evaluation of non-controlled observations. The data, although literally quantitative, are primarily used here in a qualitative sense through two-fold or multiple grouping as described by Simpson, Roe and Lewontin (1960).

Shrimp were obtained by night trawling in St. Andrew Bay, Fla., and held in a large submersible cage at the Station's Behavior Studies Facility. Recently-captured animals were used in all observations and experiments, and their total lengths recorded. All shrimp not in burrows were considered active. We arbitrarily included in this grouping all individuals that were swimming, moving about, or motionless on the substrate.

Controlled experiments were carried out with 35.6 by 20.3 by 25.4 cm. aquaria fitted with tailored plastic covers to regulate the entry of light. During the colder months, water temperature in the aquaria was partially controlled with submersible heaters. Each aquarium had about 6 cm. of washed beach sand and was filled with constantly aerated bay water ranging from 23‰ to 33‰ salinity. Observations were made hourly during 24-hour periods, using a red filtered flashlight at night.

A 38.1 by 30.5 by 30.5 cm. aquarium, contained in a light-proof black box with a light-lock viewing hood, was used for the 72-hour fatigue experiments where control of light was essential. Observations were made every two hours, and observation illumination was provided with two small red lights built into the aquarium cover.

We made non-controlled observations at the Behavior Facility in 3 to 4 meters of water, using a static underwater observation chamber (SUOC), which was previously described by Fuss and Ogren (1965). Shrimp were placed in 1-meter-square bottomless metal frame cages covered with small-mesh netting. The cages were attached to underwater viewing ports by funnel-shaped extensions of webbing on one side and were held in position by driving the cage legs into the substrate. Night illumination was by underwater flood lights fitted with red filters and controlled by switches inside the chamber. Six measured shrimp were placed in each cage and observed at hourly intervals during 24-hour periods. SUOC observations were considered as modified *in situ* observations.

Water current orientation observations were made at the entrance to St. Andrew Bay and in the vicinity of Egmont Key, Fla., using self-contained underwater breathing apparatus. A "Burrow Angle Protractor" was used to measure the angle resulting from the intersection of the water current axis and the anterior-posterior line of the burrowed shrimp. Individual measured shrimp were released about 1 m. above the bottom and each kept under surveillance by a diver until it began burrowing. The point of origin of the protractor was placed directly over the tip of the tail of the burrowed animal. The protractor was then orientated by using the current tell-tale (a piece of nylon string with a plastic stabilizer at the tip to dampen yaw) so the Y-axis of the instrument and the axis of the current coincided. The angle of burrowing was then measured to the nearest 5° by lining up the indicator arms with the anterior-posterior axis of the burrowed shrimp. Burrowing angles were recorded as degrees left or right of the water current axis when facing the current. A current velocity reading was taken as close as possible to the substrate-water interface with an Ekman-Merz type meter before and after the release of each five shrimp.

Salinity was determined by a density hydrometer or recorded continuously with an Industrial Instruments/Minneapolis-Honeywell single point strip chart salinity recorder-indicator [2] having a CELVH10270 conductivity cell. Incident light was recorded *in situ* or on the surface with a SeaTec model 210 Low Level Light Recorder with a range of 0.01076 lumens per m.^{2} to 10764 lumens per m.^{2}. Subsurface temperature measurements were taken with an Applied Research Associated Electronic Thermometer and precision grade thermometers attached externally to SUOC observation ports.

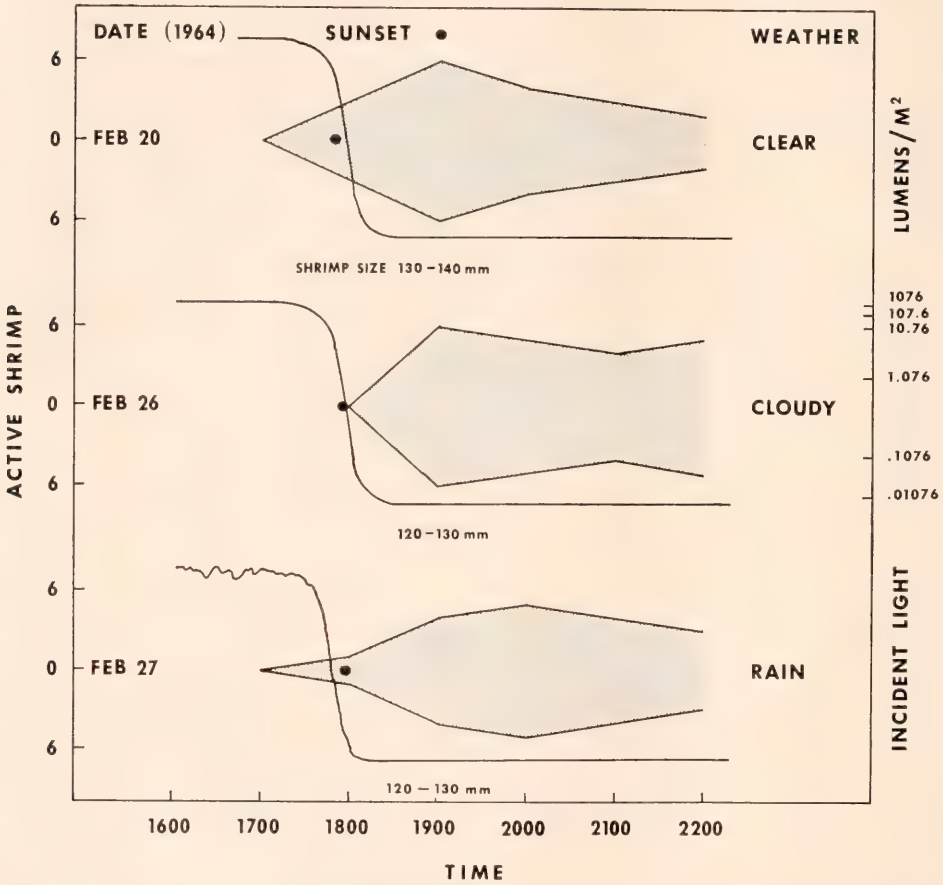

Figure 1. The emergence of burrowed shrimp in relation to decreasing *in situ* incident light. Left ordinate and shaded area = activity; right ordinate and trace recording = incident light.

For certain observations on various bottom types and during current-orientation studies, shrimp activity was recorded with either a Bell and Howell or a Bolex 16 mm. underwater motion picture camera.

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[2] Trade names referred to in this publication do not imply endorsement of commercial products.

Results

Activity Cycles

Light. SUOC and aquarium observations indicate that light is the most important single factor in the regulation of activity of adult (for this study, 80–180 mm., total length) pink shrimp. A total of 224 shrimp were observed over 24-hour periods at hourly intervals, with only 12 individuals showing any activity

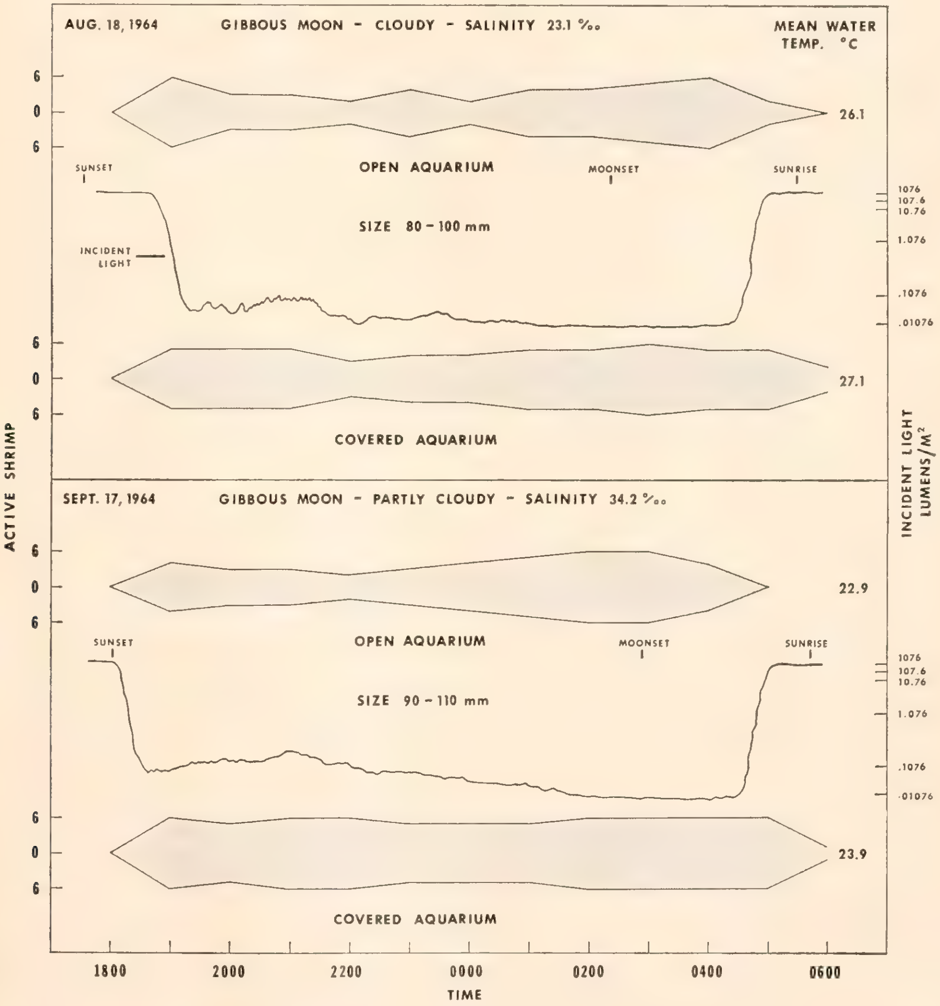

Figure 2. The effects of moonlight on the activity of pink shrimp.

during daylight hours. Of these 12, six were in one aquarium experiment when water temperature was allowed to exceed 33° C.

In the initial phases of the study, light-metering equipment was not available, but the effect of daylight was obvious. Shrimp held in cages on the bay bottom remained burrowed during the day and began to emerge around sunset, regardless of weather conditions. With the acquisition of a low-level light meter it was possible to demonstrate this burrowing activity in response to light shown in Figure 1. The sensitivity of the light meter, however, was not sufficient to detect fluctuations in light after sunset at the observation depth. By the time the shrimp were most active, about one hour after sunset, the meter showed a minimum reading and further correlation of light and activity was impossible. The early evening readings indicated that activity was fairly well established when light levels had decreased to 1.076–0.01076 lumens per m.^{2}

To test the effects of moonlight, shrimp were held in aquaria in a secluded area of St. Andrew State Park and observed hourly with a red-filtered flashlight during two periods between first quarter and full moon. The location of the observations eliminated the possible influence of extraneous light and allowed for maximum use of the light recorder. The results are shown in Figure 2 and illustrate the restrictive influence of moonlight on night activity. Maximum activity did not take place until after moonset when the meter again showed a minimum reading. These recordings, however, do show the effect of moonlight at intensities of 0.1076–0.01076 lumens per m.^{2} and help to document the relative importance of moonlight as a controlling factor in night activity. The covered aquaria in each observation series were intended as controls, but the slight decrease of activity noticeable during the early evening suggests the presence of light leaks due to improperly fitted covers.

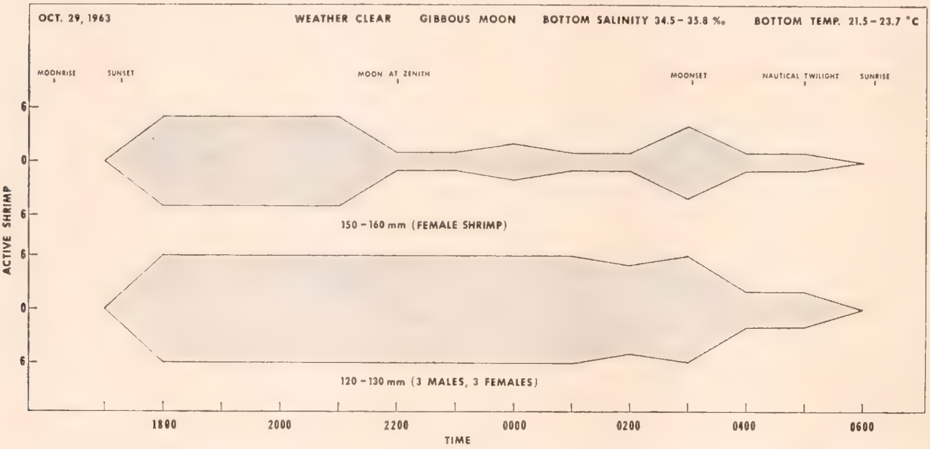

FIGURE 3. The apparent effect of moonlight on two size groups of pink shrimp at a 3-m. depth.

Figure 3 shows what appears to be a moonlight effect on relatively large female shrimp (150–160 mm.) held in a cage on the bay bottom. Although the sample is too small to generalize, size seems to be a factor in response to light, with larger individuals being the more sensitive.

Temperature. SUOC and aquaria observations showed a positive correlation between water temperature below 26° C., and night activity of pink shrimp. The effect of temperature below about 14°–16° C. seems to mask or modify the expected night behavior patterns related to light intensity noted above. Below 14° C., shrimp show a strong tendency to remain burrowed regardless of light levels or moon phase. Table I and Figure 4 show that an activity ratio of 50% or less can be expected below 14°–16° C. Figure 5 points out the degree of control exerted by low temperatures and the reversible effect of temperature fluctuations at or near 10° C. It was possible to control activity by regulating aquarium heat.

TABLE I

Random aquarium observations from November, 1963, to June, 1964, showing ratios of active to burrowed shrimp for various temperatures.
Size range: 80–175 mm. (total length)

Water temperature C.°	Active shrimp (nonburrowed)	Total shrimp observed	Activity ratio
30	5	6	0.83
29	16	18	0.89
28	21	24	0.88
27	27	30	0.90
26	27	30	0.90
25	30	36	0.83
24	39	48	0.81
23	124	162	0.77
22	112	150	0.75
21	40	54	0.74
20	47	60	0.78
19	38	50	0.76
18	55	70	0.79
17	68	94	0.72
16	112	180	0.62
15	82	168	0.49
14	24	48	0.50
13	42	88	0.48
12	25	60	0.42
11	15	60	0.25
10	10	114	0.09
9	0	42	0.00
8	0	42	0.00
7	0	30	0.00

On one occasion during a dark aquarium experiment water temperature was allowed to rise to 33.5° C. The shrimp were not actively swimming but remained motionless on the bottom and did not burrow when the hood was raised and light admitted. With decreasing temperature these animals began to burrow, but all (six shrimp, 80–90 mm.) were not covered until the water reached about 28.5° C.

An apparent size effect was also noted in the response of shrimp to low temperatures. SUOC data presented in Figure 6 show a tendency for relatively larger shrimp (larger than 140 mm.) to be less active at low temperature than smaller individuals. Figure 6 also demonstrates the rather typical and pronounced winter

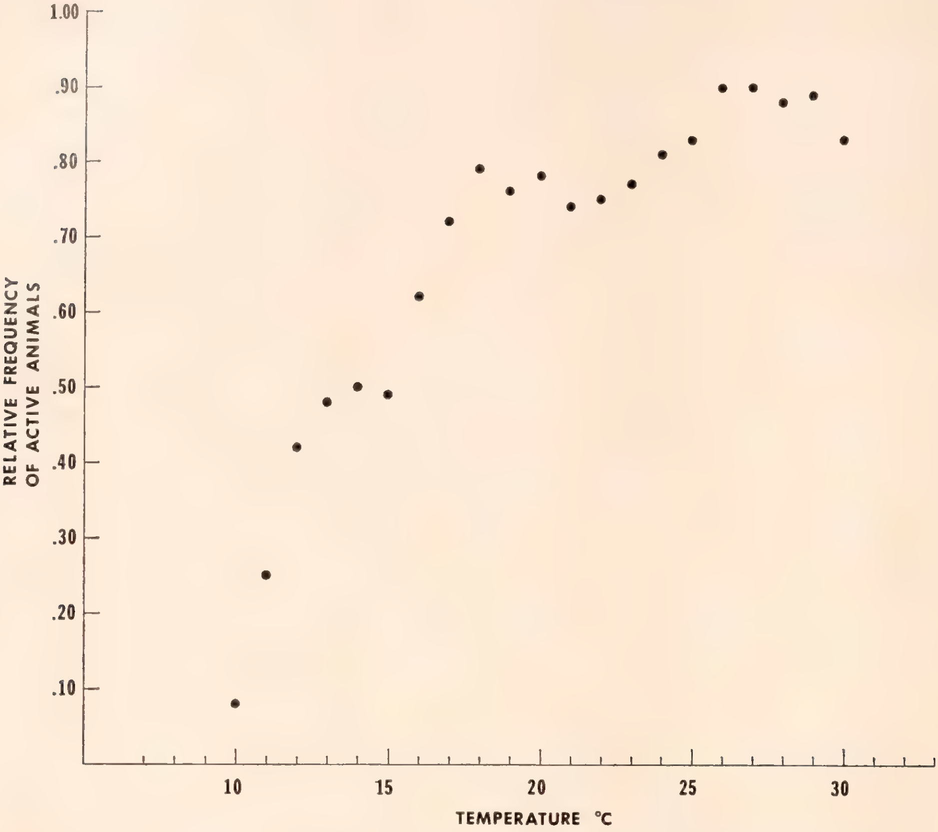

Figure 4. Relative activity of pink shrimp at various observed temperatures under controlled conditions (data taken from Table I).

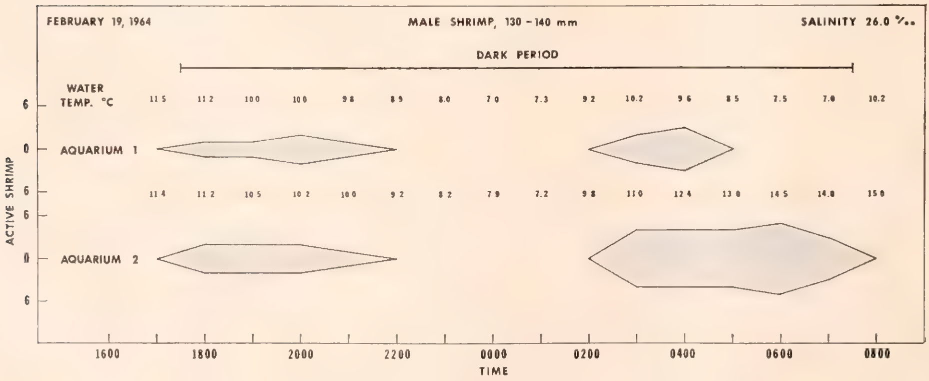

Figure 5. The effects of low temperature on shrimp activity.

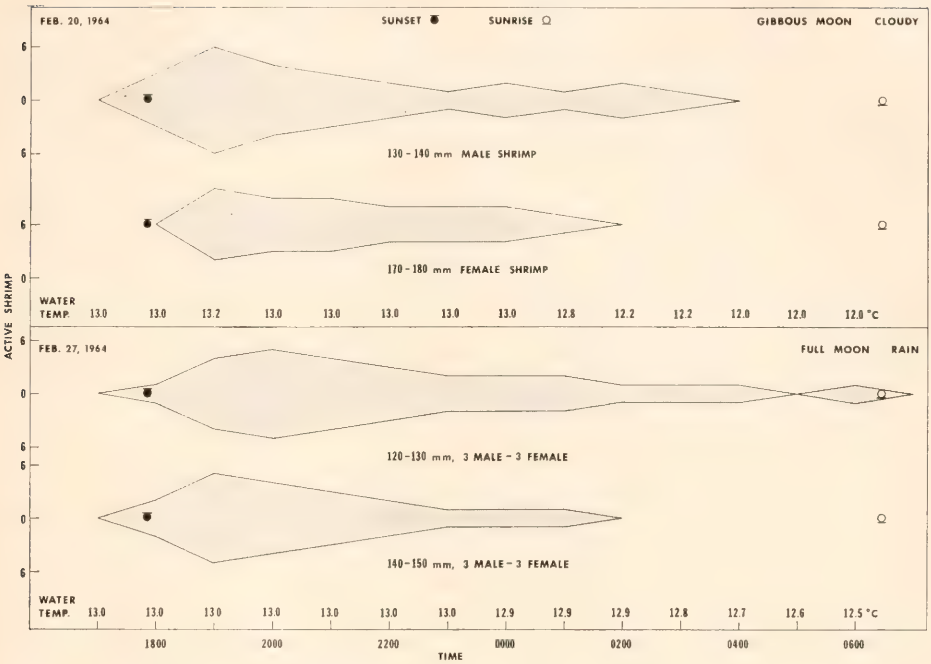

FIGURE 6. The apparent effect of low temperature on four size groups of pink shrimp at a 3-m. depth.

activity pattern of a burst of activity immediately after sunset followed by a gradual decline.

Salinity. Clear correlations were not noted between modified *in situ* activity and daily salinity fluctuations in the ranges observed (18.7–35.8‰). The largest recorded salinity excursion during any 24-hour period was 7.5‰. Salinity changes in the observation area were frequently rapid because of movements of a salt-water wedge associated with tides but did not produce corresponding changes in activity. There is a suggestion of a slight tendency for increased activity with rising salinity (Table II). Most salinity peaks, however, occurred between sunset and midnight when activity was generally greatest. Figure 7 illustrates examples of recorded salinity fluctuations superimposed on shrimp activity curves. Artificial

TABLE II

A general summary of observed events from July, 1963, to May, 1964, showing pink shrimp activity trends (general slope of activity curve) associated with salinity changes

Events	General activity trends	
	Increasing	Decreasing
Increasing salinity	13	8
Decreasing salinity	12	10

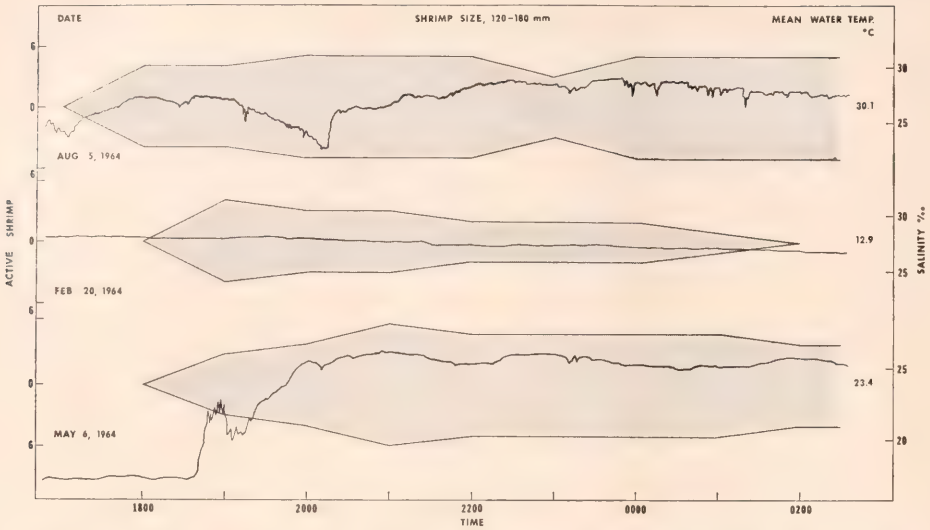

Figure 7. Shrimp activity and salinity fluctuations. Left ordinate and shaded area = activity; right ordinate and trace recordings = *in situ* salinity.

manipulation of salinity under otherwise controlled conditions was not attempted.

Fatigue. In an attempt to evaluate the behavior of pink shrimp under conditions conducive to continuous activity, experimental animals were held in a completely dark aquarium for periods of 72 hours and observed at two-hour intervals. Section A of Figure 8 shows continuous activity (non-burrowed shrimp)

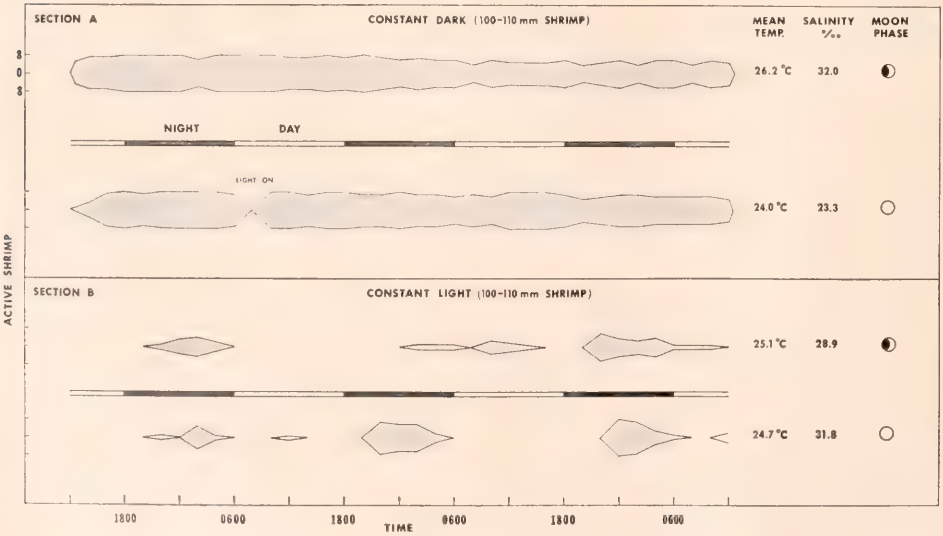

Figure 8. The effects of continuous dark and light conditions on the activity of pink shrimp.

with a gradual decline in magnitude under dark conditions. Temperature was maintained relatively constant (within 2° of the mean), and observations were kept to a minimum number. The ratios of total active individuals during each 24-hour period to the total active individuals within the observation series are given in Table III. The decline in activity noted under these conditions is attributed to fatigue. The effects of light are again illustrated by the dramatic changes in activity associated with covering and uncovering the aquaria and by the total interruption of activity due to an inadvertent lighting of the aquarium between 06 00 and 08 00 hours on the second day of the second series.

Availability of food. The effects of food availability on the diel activity of non-starved pink shrimp seem to be of little consequence. Twenty-four-hour aquaria observations on the activity of fed (with chopped mullet) and non-fed shrimp showed no obvious differences. Ratios of activity (activity for one group/activity for both groups) for 120–130 mm. fed to non-fed shrimp were 0.52 to 0.48, respectively, under conditions of constant salinity (23.0‰) and relatively constant temperature (mean temperature 20.2° C.).

TABLE III

Activity ratios of shrimp under constant dark and light conditions

<table>
<tr><th rowspan="2">24-hour periods:</th><th colspan="6">Activity ratios</th></tr>
<tr><th colspan="2">1st</th><th colspan="2">2nd</th><th colspan="2">3rd</th></tr>
<tr><td>Constant dark</td><td colspan="2">0.36</td><td colspan="2">0.34</td><td colspan="2">0.29</td></tr>
<tr><td>Constant light</td><td colspan="2">0.20</td><td colspan="2">0.32</td><td colspan="2">0.48</td></tr>
<tr><th>Diel activity:</th><th colspan="3">Day</th><th colspan="3">Night</th></tr>
<tr><td>Constant dark</td><td colspan="3">0.48</td><td colspan="3">0.52</td></tr>
<tr><td>Constant light</td><td colspan="3">0.16</td><td colspan="3">0.84</td></tr>
<tr><th>Moon phase:</th><th colspan="3">Full</th><th colspan="3">Last Quarter</th></tr>
<tr><td>Constant dark</td><td colspan="3">0.51</td><td colspan="3">0.49</td></tr>
<tr><td>Constant light</td><td colspan="3">0.56</td><td colspan="3">0.44</td></tr>
</table>

To test the effect of available food on starved shrimp in relation to light, food was placed in aquaria at the end of 72-hour observations (Fig. 8). At the times food was introduced, all shrimp were burrowed and the aquaria lighted. In the dark series, ending October 2, 1964, one individual deburrowed 10 minutes after the addition of food and fed for 5 minutes before reburrowing. On October 23, 1964, two individuals deburrowed and began to feed 15 minutes after food was added. Within two minutes one of these reburrowed and a third emerged to feed. These two individuals remained active and feeding for about 30 minutes. On October 30 and November 20, 1964, after 72 hours of continuous light and little activity, only one shrimp emerged from his burrow to feed.

Diurnal cycles. Diel periodicity due to solar stimulus is obvious, and the reactions of pink shrimp to artificial light and moonlight strongly suggest external

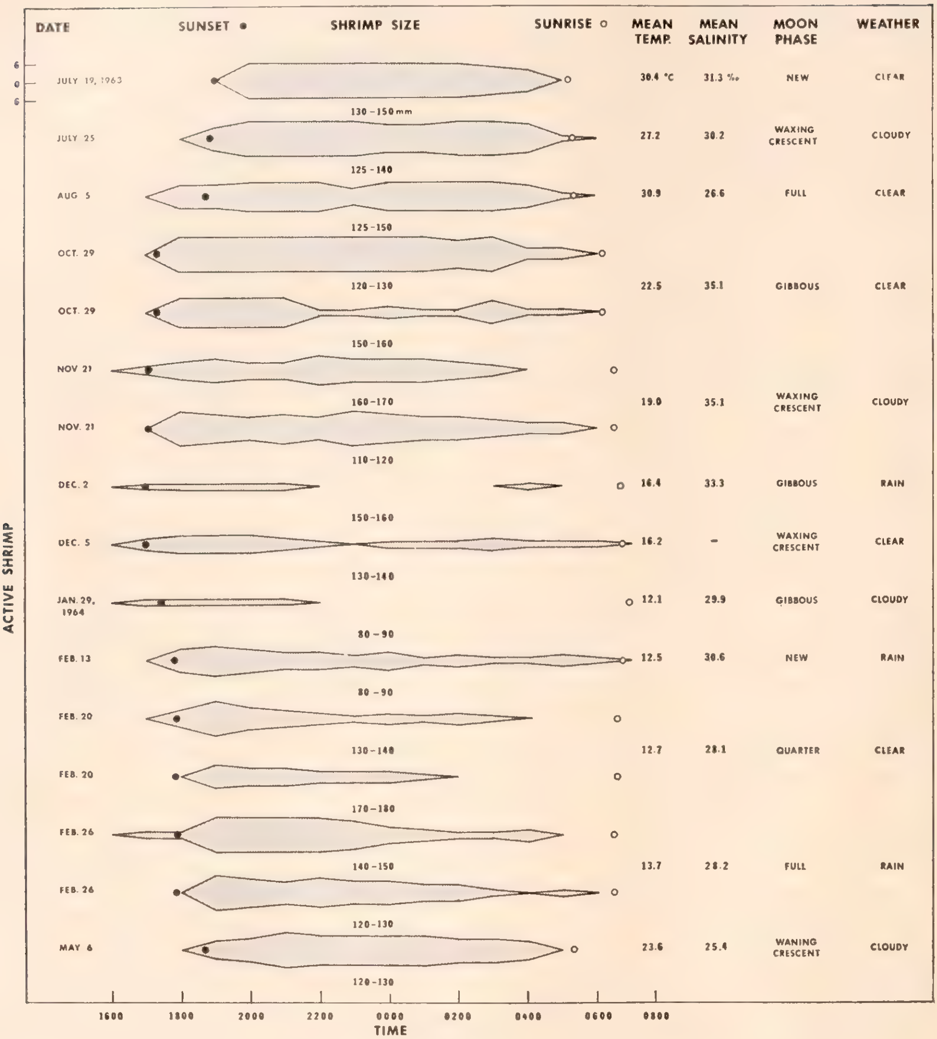

FIGURE 9. Seasonal activity cycles of selected groups of pink shrimp.

control as the primary factor. However, an endogenous component is indicated by the data presented in Section B of Figure 8. In these series, under conditions of constant light (107.64 lumens per m.^{2}) and fairly constant temperature (within 2° of the mean), a somewhat persistent nocturnal rhythmicity is detectable. The evidence for this circadian rhythm is sketchy, but is considered worth reporting in the light of previous work on other penaeid shrimp (Dall, 1958; Racek, 1959). The ratios of all active animals for day- to night-activity are given in Table III and show the disparity between constant light and dark conditions. An increase in activity for each 24-hour period during the constant light series is also shown in Table III. The tendency for increased activity under lighted conditions with the passage of time was noted during other shrimp studies at this station when animals were held in lighted aquaria for long periods.

Lunar Cycles. Direct correlations between lunar cycles *per se* and nocturnal activity were not well defined. Aquarium observations under constant dark and light conditions (Table III) during full and last quarter moon phases did not show strong evidence of lunar rhythmicity. Non-controlled observations from July, 1963, to May, 1964, show that 53.7% of 60 animals observed were active during the lunar phase of last quarter-new-first quarter and 44.4% of 36 animals observed were active during the first quarter-full-last quarter phase. The summary presented above was taken from observations with water temperatures above 15° C., and reflects activity between sunset and sunrise.

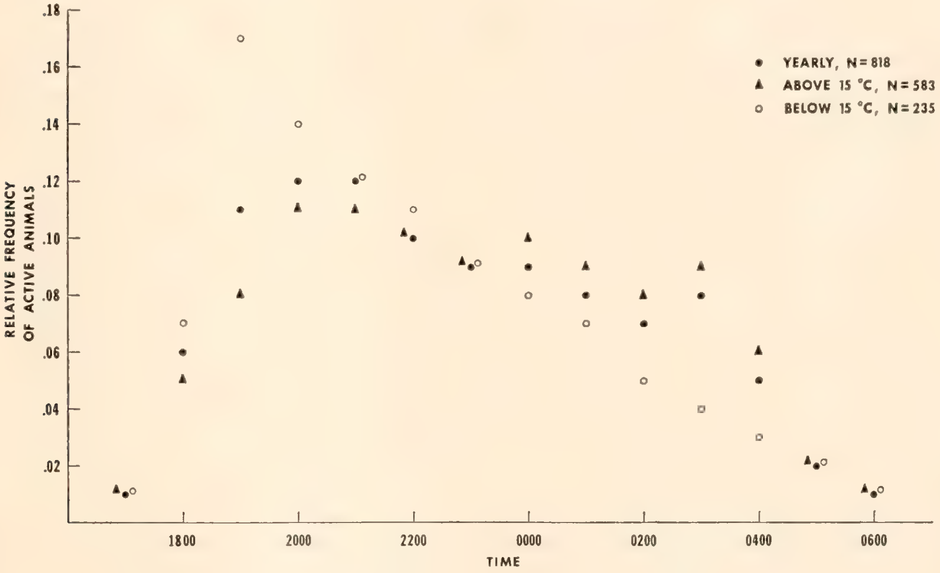

FIGURE 10. Nocturnal activity patterns reflecting photoperiod and temperature variances of the seasonal cycle.

Seasonal cycles. Figure 9 illustrates the seasonal activity cycles of selected groups of pink shrimp under uncontrolled conditions at a depth of 3 m. The duration of activity seems to be primarily controlled by photoperiods and the proportion of shrimp active at any one time by bottom water temperatures. Long winter nights are characterized by extended activity periods and a decrease in the number of active animals. Summer nights are characterized by an increase in the number of shrimp active over a shorter period. Figure 9 also indicates the ability of pink shrimp to acclimate to low water temperatures. When winter temperatures first dropped to about 16° C., (mean temperature for observation period), activity was sharply reduced. As low temperatures continued over a few months, more activity was noted even at means of 12° to 13° C.

Figure 10 shows the relative frequency of active shrimp at given time intervals to the total activity observed during all non-controlled phases of this study. The cumulative seasonal data point to an activity peak around 20 00 to 21 00 hours, but show a considerable disparity between summer and winter nocturnal activity patterns. The difference between the two temperature groupings of animals at 19 00 (Fig. 10) could be the result of earlier sunsets during the cold season (shortened photoperiod). At temperatures below 15° C., activity data suggest a relative relationship between light and temperature. Immediately after sunset the animals become very active in the absence of light and then gradually respond to temperature and possibly fatigue by burrowing.

WATER CURRENTS AND BURROWING ORIENTATION

Observations of the effects of bottom water currents on the orientation of burrowing shrimp show that a positive rheotaxis may be expected at current velocities above about 0.2 m. per second on level bottoms. Table IV indicates that burrowing with respect to water current is fairly random below 0.2 m. per second with positively orientated individuals showing a mean burrow angle of 40° to the current axis. Above 0.2 m. per second at least 90% of the burrowing shrimp orientated positive to the current, and the mean burrow angle decreased. As water currents increased there was a tendency for shrimp to burrow more nearly in line with the current axis. The data show that between 0.44 and 0.54 m. per second, 63% of the animals observed burrowed within 25° of the bottom current axis. At current velocities above 0.2 m. per second the ratio of shrimp burrowing to the right and left of the current axis was 0.52 to 0.48, respectively. Orientation to the right or left of the current axis may therefore be considered random.

TABLE IV

Shrimp orientation to current velocities

Current speed (m. per second)	Total no. of shrimp	% Positive orientation	% Orientation within 25° of current axis	Mean positive orientation to current axis (degrees)	*Positive orientation on either side of current axis	
					Left	Right
0.44–0.54	30	90	63	21°	12	15
0.20–0.39	35	94	45	28°	17	16
0.06–0.19	35	57	25	40°	8	12

* Individual shrimp.

During orientation studies shrimp were occasionally observed to maintain their position relative to the bottom by swimming against currents in excess of 0.26 m. per second with the pleopods providing the propulsion.

BOTTOM TYPES

Observations on the effects of bottom types in relation to burrowing were random and qualitative and made during cruises of the R/V *George M. Bowers* to various locations in the southern and eastern Gulf of Mexico. Captured shrimp were released above the bottom during daylight hours, and burrowing activity was recorded with an underwater motion picture camera.

The bottom type seemed to affect the rate of burrowing more than the degree of burrowing, with shrimp penetrating all substrates observed. Shrimp were capable of burrowing in extremely hard substrates of sand and sand-shell, and entered soft bottoms in a matter of seconds. On heavy "grass" (*Thalassia testudinum* and *Syringodium filiforme*) bottoms, adult shrimp occasionally experienced difficulty in reaching the substrate. Once on the bottom, however, the animals were able to burrow among the plant rhizomes with little effort.

Discussion

The nocturnal habits of pink shrimp have been observed and recorded in a number of earlier studies (Idyll, 1950; Viosca, 1957; Williams, 1958; Eldred, 1958; Eldred *et al.*, 1961; and Fuss, 1964), and fishermen have learned that the best catches are taken during the hours of darkness. Pink shrimp usually remain burrowed during the day, and the reason is apparently protection. Observations by swimmers during the course of this and previous work reveal that penaeid shrimp are relentlessly attacked by a variety of small and large fishes when exposed in relatively clear water. Great numbers of small pinfish (*Lagodon rhomboides*) were frequently observed attacking single large adult pink shrimp. The small fishes, often no larger than the shrimp, would immobilize their prey by biting off the appendages. Motion picture records of these attacks show the shrimp attempting to escape by burrowing.

Laboratory manipulations by other investigators concerning the effect of artificial light on activity and burrowing are somewhat contradictory. Eldred (1958) noted that during night observations hard-shelled *P. duorarum* held in aquaria immediately burrowed in the sand when exposed to green, white, and yellow lights. Aaron and Wisby (1964), in their studies of pink shrimp exposed to a 3.23 lumens per m.^{2} white light source in an experimental apparatus, report a positive phototaxis. The results of our study show an inhibition of activity or burrowing response in the presence of light which would be expected if previous field observations (Viosca, 1957; Eldred *et al.*, 1961; Fuss, 1964) are correct. The apparent discrepancy between investigators may be due to animal size, development, and sexual maturity, or the intensity of the light used. Shrimp observed by Eldred (1958) were 82 to 102 mm., mature individuals, and in this study the animals ranged from 80 to 180 mm. Aaron and Wisby used shrimp of approximately 55 to 105 mm. ("between the leading edge of the blades and the spine of the telson"—size estimate made from Figure 2 of their paper) and state that specimen size played an important part in photic response. They showed that photoactivation (animal moved toward the light) was greatest in shrimp measuring 75 mm. and photoactic drive (animal moved through an alternating current electrical field toward the light) was least in 90-mm. specimens. Eldred *et al.* (1961) found that individuals smaller than 55 mm. were observed to be active when exposed to bright daylight or electric lights, whereas larger specimens remained burrowed.

The results of this study indicate that larger shrimp are more sensitive to light than smaller shrimp although most individuals within the size ranges observed showed a negative photic response (burrowed in the presence of daylight or artificial light). Korringa (1957) cites examples of animals that are positive to light at one level of illumination and negative at other levels. This phenomenon, however, as a possible explanation of the discrepancy outlined above is generally unsatisfactory, as Aaron and Wisby report a positive response to 3.23 lumens per m.^{2} while our results (modified *in situ*) indicate a negative response to the same value. However, Pardi and Papi (1961) state that a great many crustaceans react either positively or negatively, depending on light intensity. According to Ghidalia and Bourgois (1961) certain penaeids (*Parapenaeus longirostris, Aristeus antennatus, Aristeomorpha folicaea*) of the Western Mediterranean show a positive phototaxis in weak lunar light and negative phototaxis in intense solar light. Further detailed experiments may provide more concrete conclusions.

The most logical explanation of this variable photic behavior would seem to be size or age because photopositive reactions are generally attributed to smaller animals (below about 80 mm.) and photonegative responses to larger individuals. Pardi and Papi (1961) report that ontogenetic changes in light responses are common in crustaceans, with a greater photopositive tendency in younger stages. Ghidalia and Bourgois (1961) also noted that young shrimp usually exhibited a marked positive response, but adults showed one that was mild or even negative. The probable increased sensitivity of larger shrimp to light suggests an adaptive process which may be a prerequisite to survival in a hostile environment. Pink shrimp may well become more responsive to stimuli when they move from the protected sea grass environment of the juvenile to the open water habitat of the adult. The results of this study indicate that when males and females are about the same size there is little difference in response to light. As all of the very large specimens (170–180 mm.) were females, the apparent increased sensitivity of females is probably due to this disproportionate sex-size distribution.

SUOC observations and fishing experience leave little doubt that adult *P. duorarum* reacts negatively to solar light and generally remains burrowed during the day. The data presented here also strongly indicate that moonlight plays an important role in nocturnal activity. It is reasonable to assume that lunar light could directly influence night activity if the light threshold estimate (10^{-5} to 10^{-6} lux) cited by Waterman (1961) for crustaceans in general is valid for *P. duorarum*. The early evening burst of activity noted in SUOC observations may be associated with a period of dark-adaptation followed by increased light-sensitivity related to superposition vision and a consequent decrease in activity. Waterman (1961) reports that dark-adaption in certain crustaceans, including *Cambarus* and *Homarus*, takes about 20 minutes to an hour. The amount of moonlight penetration at depths equal to the maximum depth range (about 68 meters, Springer and Bullis, 1954) of *P. duorarum* in sufficient quantity above the estimated minimum threshold seems likely, at least under favorable weather conditions. Clarke and Wertheim[3] (1956) reported a light energy reading of about 0.001 micro watt per cm.^{2} at a depth of 100 m. in the Florida current at 20 30 hours under half-moon conditions. This reading is approximately equivalent to 0.0014 lux (Roesel, 1964, personal communications) if the energy spectrum is assumed to equal that shown by Sverdrup, Johnson and Fleming (1942) for a depth of 100 m. in clear oceanic water. Even if the usual habitat of *P. duorarum* is more turbid than the Florida current, the full moon probably provides sufficient light in most cases to influence activity. If the estimated minimum response of the shrimp's eye is 10^{-6} lux and the estimated maximum night light level at a depth of 100 m. is 1.4×10^{-4} lux, a response to moonlight can probably be expected. Extreme conditions of weather or turbidity would undoubtedly modify moonlight effects.

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[3] Clarke and Wertheim also gave data on light energy measurements in turbid slope water but conversion to an approximate unit of illumination is impractical due to lack of spectra data.

If light is the most important single factor in the regulation of pink shrimp activity, then it is necessary to explain why occasional day catches exceed night catches when turbid water is encountered. Eldred *et al.* (1961) and Fuss (1964) provide an explanation for this obvious inconsistency.

The influence of temperature on burrowing habits of *P. duorarum* has been suggested by Williams (1955) and Eldred *et al.* (1961), who agreed that shrimp probably remain buried in the substrate during periods of cold. Eldred has suggested that the substrate temperatures are warmer than those of the surrounding water and burrowing serves as a protective measure in the survival of the species during periods of extreme cold. Data presented here substantiate this conclusion and indicate that sudden temperature changes that occasionally occur in shallow water may cause burrowing rather than migrations to deeper water. Migrations of pink shrimp into deeper waters to avoid cold are discussed by Tabb, Dubrow and Jones (1962).

Gunter (1957) reviewed the effects of temperature on marine organisms and stated that (p. 166) "within normal limits the absolute temperature seems to have less effect than temperature changes." His statement holds true for *P. duorarum*. Early winter low temperature produced significant reductions in activity at levels which seemed to have little effect in late winter.

Aldrich (1964, personal communications) found a relation between the size of *P. setiferus* and low temperature sensitivity, with larger animals being seemingly less tolerant. Our data suggest a similar relation as evidenced by modified *in situ* observations on the comparative activity of large and small (relative size) shrimp under identical temperature conditions.

The complete cessation of activity at temperatures below 10° C. is supported by data presented by Eldred *et al.* (1961) and Williams (1955). Severe reductions in pink shrimp activity below approximately 14°–16° C. were found by Williams (1955) and by Dall (1958) for *Metapenaeus mastersii*. Aldrich (1964, personal communications) also found *P. setiferus* was adversely affected by temperatures below 12°–14° C. In general it seems that the lower temperature limit for penaeid shrimp activity is about 14° to 16° C.

Eldred *et al.* (1961) have also suggested that, as a protective measure, *P. duorarum* may burrow into the substrate during extremely high summer temperatures. Our limited observations, however, indicated that high temperatures (above about 33° C.) cause pink shrimp to deburrow, but there is a possibility that the reason was actually oxygen deficiency due to the increased water temperature. Egusa and Yamamoto (1961) have shown that *P. japonicus* projects its respiratory siphon above the substrate surface when environmental oxygen is reduced to 1.0 cc. per liter and came out of the bottom entirely when 0.5 cc. per liter is reached. Further work is needed on the effects of high temperatures and low oxygen tensions on shrimp burrowing behavior.

It is not surprising that salinity fluctuations within the ranges observed did not produce a marked effect on pink shrimp activity. Hoese (1960) and Gunter

(1961) disagree as to the euryhaline characteristics of *P. duorarum*, but the minimum salinities recorded in our study were considerably above the lower limits in question. Zein-Eldin and Aldrich (1964) have reported the ability of grooved postlarval penaeids (very probably *P. aztecus*) to withstand wide fluctuations of salinity, and Tabb *et al.* (1962) concluded that *P. duorarum* is tolerant to wide salinity ranges, but less so as adult size is reached. The osmoregulatory powers of *P. duorarum* have been verified by Williams (1960), who demonstrated that pink shrimp can maintain themselves fairly well for limited periods in salinity ranges of 10‰ to 30‰.

Pardi and Papi (1961), however, state that lowering the salinity of the medium generally results in photonegative reactions by marine crustaceans, and Racek (1959) concluded that prawn stocks in estuaries bury themselves when salinities decrease rapidly because of heavy rainfall. These conclusions give support to the suggested increase of activity with rising salinity noted in this study.

The results of fatiguing experiments indicate that pink shrimp are capable of extended activity under certain conditions of light and temperature, although the data do not provide information on the endurance of individual animals. Feeding trials at the end of 72-hour observation series also indicate that feeding requirements after a limited period of starvation are not sufficiently critical to overcome usual or expected diel periodicity established by photoperiods. Dall (1958) reports that *M. mastersii*, which is sensitive to light, also refused food during the day and remained burrowed. Results of continuous light observations suggest that conservation of activity will extend the ability of *P. duorarum* to undergo periods of starvation.

Other studies previously cited show *P. duorarum* is nocturnal, as did the results of earlier observations at this station (Fuss, 1964). As Brown (1961) pointed out (p. 401), "organisms dwell in a rhythmic environment," and there are many 24-hour cycles that are associated with conspicuous changes in marine habitats. The daily activity patterns of pink shrimp seem to be primarily controlled by photoperiod and other modifying environmental factors, but there is some evidence of an endogenous component. There are many excellent and exhaustive works on environmentally controlled periodicity and circadian rhythms, including those of Brown *et al.* (1955a, 1955b), dealing with fiddler crabs, in which they suggest responses to a complex of environmental stimuli and endogenous factors, and Mori's (1960) studies of the sea-pen *Cavernularia obesa*, which seems to perform rhythmic activity under the control of both environmental and physiological factors. The activity of *P. duorarum* is undoubtedly influenced by many factors and is complex in nature.

It is interesting to note that *P. duorarum* did not show a diel periodicity under controlled dark conditions, but did show some pattern of activity under constant light conditions. A similar phenomenon was recorded by Welsh (1930) in studies on the migration of distal pigment cells in the eyes of two fresh-water shrimp of the genus *Macrobrachium*. He found a persistent diurnal rhythm under constant illumination, but noted that animals kept in the dark showed no movement of the distal pigment cells. Brown (1961) has suggested a possible explanation related to the production of dark-adapting hormones as the regulatory agents that may apply here. In total darkness, fluctuations in dark-adapting hormones would not produce changes in dark-adapted eyes. There is a possibility that the persistent rhythm component of *P. duorarum's* daily activity cycle could be related to dark- and light-adapting hormones. Dall (1958) recorded a persistent daily rhythm in the gross activity of *Metapenaeus mastersii*, as did Racek (1959) for *Penaeus plebejus*.

The increased activity with time under conditions of constant light is probably an acclimation to light rather than a quest for food, as the animals refused to deburrow when food was introduced at the end of the series. Pardi and Papi (1961), in their review of kinetic and tactic response in crustaceans, report that light adaptation affects the sign of light responses in a number of animals, and Schöne (1961) describes conditioned learning in *Palaemonetes* and other forms.

Data derived from this study show that cessation of night activity is not as well defined as the beginning. Wheeler (1937) describes similar patterns in the swarming behavior of certain prawns, including *Penaeus brasiliensis,* at the Bermuda Biological Station. As Wheeler's work was done before Burkenroad's (1939) differentiation of the *P. brasiliensis* complex, it is possible that the animal observed was actually *P. duorarum* (Gunter, 1964, personal communications). The reason for this pattern would seem to be a modifying influence of other factors, such as temperature and fatigue, on a primarily light-controlled evolution. The results of this study agree with Wheeler's (1937) conclusion that the direct action of light is the strongest inhibiting factor in diel activity of prawns, at least for this species.

Many fishermen believe that pink shrimp catches are poorer during full moon periods (Iverson and Idyll, 1959), and earlier *in situ* observations (Fuss, 1964) gave support to this theory. Our preliminary studies during the summer of 1962 were made under near-optimum water conditions for light penetration during clear weather. The present results concerning moon phase correlations are based on SUOC observations. The results are not well defined; however, they are based on much more extensive investigations, including observations in foul weather, during low bottom temperature, and in high turbidity. The studies lead to the conclusion that any moon phase correlations are directly related to light intensity and consequently are dependent on weather and turbidity.

Eldred *et al.* (1961) reported that moon phase showed no effect on the abundance of shrimp in samples, but Aaron and Wisby (1964) found that moon phase had a significant effect on the photoactivation of shrimp and that maximum photoactivation occurred during the full moon and the minimum during new moon. Our controlled observations suggest a slight activity correlation with full moon periods, but the results are not conclusive.

It would not seem unreasonable to assume that nocturnal pink shrimp are affected by moonlight if not moon phase *per se*. As Korringa (1957) has pointed out, the rhythm of the moon must be of utmost importance to marine animals that hide in their dark shelters during daylight hours, and Fox (1924) has also suggested that additional illumination on certain nights of the month, although weak, could cause shorter or longer periods of activity. Wheeler (1937) concluded that the coincidence of prawn swarming with the new moon is so marked that some form of correlation with the changing phase of the moon is beyond question. He suggests that the direct action of light is the clue. Racek (1959) has objected to this conclusion on the grounds that it is unlikely that the cause of periodicity in behavior of predominantly demersal prawns is the direct inhibitory effect of light. Under conditions of extensive turbidity or inclement weather his argument is probably valid, but data presented by Clarke and Wertheim (1956) would seem to indicate that moonlight may very well control the activity of certain penaeids.

The seasonal cycles observed in this study and related to photoperiod and temperature resemble the pattern presented by Wheeler (1937) for swarming *Anchistoids.* The shift from a near bimodal summer cycle of activity to a sharp unimodal winter pattern is probably a direct result of temperature, but other factors may be involved.

Apparently no data are published on the swimming speeds or responses to water currents of penaeid shrimp. The positive orientation to water currents in excess of about 0.2 m. per second by *P. duorarum* seems to be a mechanical phenomenon, but probably also depends on optic fixation, as Hadley (1906) demonstrated with the American lobster, *Homarus americanus.* Although a detailed evaluation of the fluid dynamics associated with burrowing orientation is beyond the scope of this paper, we may assume that shrimp in contact with the bottom have a general streamlined form and show certain expected characteristics of such shapes in a fluid medium. As the water velocity increases, the shrimp should burrow more nearly parallel to the current axis and thus reduce the angle of attack and drag coefficient. When the angle of attack exceeds a certain critical value, drag increases greatly (Shapiro, 1961) and the animal experiences difficulty in maintaining position on the bottom. The angle of attack is seen to decrease with increased current velocity as reflected by the shrimp mean burrow angle.

Our observations on the burrowing ability of *P. duorarum* on various bottom types agree with previous studies (Williams, 1958; Hildebrand, 1955), and confirm the animal's ability to enter extremely coarse substrates. In one case in particular, off Panama City Beach, pink shrimp were observed to burrow in an extremely hard sand substrate in which it was difficult to force a diving knife. The animal seems to use a hydraulic mechanism in the burrowing process on hard substrates. Abdominal muscles were occasionally flexed after initial penetration, apparently forcing entrapped water with suspended sediment from beneath the burrowing shrimp.

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SUMMARY

1. The effects of certain environmental and physiological factors on the activity and burrowing habits of pink shrimp, *Penaeus duorarum,* are described.

2. Adult pink shrimp have a tendency to burrow in the presence of solar light and maximum activity does not occur until incident illumination is below 0.01076 lumens per m.^{2} Light is apparently the most important inhibiting factor in diel periodicity.

3. Moonlight affects the nocturnal activity of pink shrimp to a limited extent but the inhibiting effects may be modified by weather and water turbidity.

4. There is a general inverse relation between nocturnal activity and water temperatures below about 26° C., and maximum activity seems to be associated with temperatures of 26° to 27° C. At water temperatures below 14° to 16° C. less than half of the animals will be active and all activity ceases below about 10° C. Shrimp apparently deburrow at temperatures above about 33° C. but remain quiescent.

5. An increase in activity with rising salinity is suggested but effects of salinity within the ranges observed (18.7–35.8‰) are probably insignificant.

6. Shrimp are capable of fairly extended activity and fatigue is probably not a limiting factor until after about 48 hours of continuous activity.

7. The need for food after limited starvation (72 hours) does not seem to overcome the activity-inhibiting effects of sunlight; and conservation of energy by burrowing will apparently extend the ability of pink shrimp to undergo periods of starvation.

8. Pink shrimp are capable of limited acclimation to light and low temperature, and large animals (above about 140 mm.) are apparently more sensitive to these factors than smaller ones.

9. Cycles of activity related to photoperiods are well defined and there is evidence of an endogenous component in the diel periodicity of pink shrimp. Persistent circadian rhythms, however, are only discernible under conditions of constant light.

10. Correlations between activity and lunar cycles *per se* are not well defined.

11. Seasonal cycles of activity seem to be primarily controlled by photoperiod and temperature. Peak daily activity during a seasonal cycle occurs at about 20 00 hours.

12. Burrowing shrimp show a positive rheotaxis in water currents at velocities above about 0.2 m. per second and as velocity increases, their burrowing angle to the current axis decreases. Orientation to the left or right of the current axis seems to be random.

13. Pink shrimp are capable of penetrating very coarse bottom types including hard sand, sand-shell, and substrates overlaid with heavy plant growth.

14. Burrowing serves as a protective measure against predators and adverse environmental conditions.

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RESPIRATION DURING THE REPRODUCTIVE CYCLE IN THE SEA URCHIN, STRONGYLOCENTROTUS PURPURATUS[1]

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During the reproductive cycle of the purple sea urchin, *Strongylocentrotus purpuratus,* the gonad may grow many-fold within a few months while the other organs show little relative change (Bennett and Giese, 1955; Giese, 1959). The bulk of the sea urchin tissue is found in its body wall, gut and gonad. The gonad of a non-gravid (resting or spent) sea urchin may have less organic matter than its body wall or gut, but the gonad of a gravid animal has about as much organic matter as all of its other tissues combined (Giese, 1966). Increase in the respiratory rate of the sea urchin corresponding to the increase in gonad size may thus be expected if the tissue O_2 demand remains uniform and O_2 supply does not become limiting. A survey of the literature indicates that variations in respiration correlated with the reproductive cycles of echinoderms have not been investigated (Farmanfarmaian, 1966).

To determine the relationship between the rate of respiration and the reproductive state, the respiratory rate was measured on sea urchins of different gonad indices (ratio of gonad to body wet weight × 100) taken at various times during the annual reproductive cycle (Bennett and Giese, 1955). Similar measurements also were made on specific body components (body wall, lantern, perivisceral fluid, gut and gonads) of the animal.

Materials and Methods

The sea urchins were collected near Yankee Point (5 miles south of Carmel, California) and at Moss Beach, Pescadero, Pigeon Point, and Pillar Point, California. They were placed in constantly filtered and aerated sea water in the 13° C. constant-temperature room (at Stanford) or in running sea water at ambient sea water temperature (at Pacific Grove). The animals were not fed in captivity. For studies on whole animals it was necessary to pick out individuals that could be placed inside the respirometric flasks (4 cm. diameter); animals of similar size were also used for studies on body components. The urchins used were about 3.5 cm. in test diameters and varied between 13 and 26 gm. in wet weight. They were placed individually in large respirometric flasks and covered with about 15 ml. sea water. In general, standard Warburg-Barcroft respirometry was employed (see Farmanfarmaian, 1959). Occasionally a sea urchin in a respirometric flask lifts a portion of its surface above sea water. Consequently the Q_{O_2} values for entire sea urchins may on such occasions be somewhat higher than expected because respira-

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tion is higher in air than in water (Farmanfarmaian, 1959). Urchins were watched when possible during experiments and note taken of movement out of water; such data were not used.

Body components were obtained from urchins 3 to 6 cm. in test diameter and 15–26 gm. in wet weight. To remove all debris resulting from excision of body components, each piece was washed 3 to 6 times in filtered sea water. Each piece of body component (about 0.2 to 1.0 g. wet weight, depending upon the tissue) was weighed (Mettler balance), and placed in the Warburg flask in 2 to 4 ml. filtered sea water.

The perivisceral fluid bathing the tissues of an echinoderm is essentially similar to sea water in constitution (Robertson, 1953). In a number of trials the respiration of equal aliquots of body components was found to be the same in perivisceral fluid and in sea water. Consequently, for simplicity, in all subsequent experiments the body components were placed in filtered sea water for respirometry.

With the precautions noted above no bacterial growth was observable over 2 to 5 hours of experimentation with either whole animals or body components. In all experiments reported the respiratory rate (a rising straight line plotting total respiration against time) remained constant for the duration of measurement. This further confirms that oxygen uptake due to bacterial growth was not significant. Because cutting may liberate nutrients from body components, antibiotics (15 γ streptomycin and 4 γ penicillin per ml.; see Skinner, 1962) were tested in sea water. No difference in respiration was observed between samples of the same body components with and without antibiotics. Therefore, their use was discontinued.

All manometric calibrations were performed by injecting a known volume of air from a calibrated syringe. Since the amount of tissue or size of the urchin differed in each experiment, calibrations were made at the beginning or conclusion of each experiment.

To obtain the rate of respiration per unit nitrogen, entire sea urchins were digested with sulfuric acid and the nitrogen content of an aliquot of the digested urchin was determined by the micro-Kjeldahl using the Markham distillation method (Markham, 1942). In practice, each sea urchin was drained on a towel and weighed. It was then opened and the perivisceral fluid drained, measured and dried. Next, the gonads were removed and their wet weight determined. The body wall, Aristotle's lantern, and the gut (washed free of contents) were also drained, weighed wet, and dried. Finally, all these tissues were combined, ground and aliquots were digested. The total nitrogen has been taken as a measure of the tissue content of an animal (Zeuthen, 1947).

All the sea urchins used in the latter part of the present study on whole animals were starved for several days to several weeks before use. Starvation was necessary because urchins fed to satiation defecate continually, sometimes even after two days of starvation. The bacteria in the fecal material would make respiratory determinations meaningless. Urchins from which body components were to be removed for respirometry were also comparably starved. The respiratory rates of the urchins (and tissues) are lower than those previously reported (Farmanfarmaian and Giese, 1963), possibly because starvation decreases the respiratory rate in *S. purpuratus* (Farmanfarmaian, 1966). Desirable as control of nutrition might have seemed, it was not possible to feed the urchins at Stanford because of the large volumes of sea water required for daily cleaning. Another factor which may determine the Q_{O_2} level is temperature acclimation, which occurs in a few days (Farmanfarmaian and Giese, 1963). The urchins are kept in a constant temperature (13° C.) at Stanford. They are subjected to higher and varying temperatures at the Marine Station. The data for animals studied at each of the laboratories are, however, relatively consistent.

RESULTS

1. *Rate of respiration of whole sea urchins of different gonad indices*

The total nitrogen in a sea urchin of a given size doubles during the growth of the gonad from the immature state to the fully gravid condition, indicating that the tissue in the sea urchin has doubled. Yet, as seen from Figure 1A, there is essentially no change in the respiratory rate per unit wet weight when the gonad index increases from 3 to 20. For a sea urchin of a given size and weight this rate is about constant regardless of its gonad index. The increase in size of the gonad does not entail a proportional increase in total wet weight of the sea urchin, because as the gonad grows, the amount of perivisceral fluid remaining inside the test decreases.[3] Since the specific gravity of the gonad is only slightly greater than that of the perivisceral fluid, increase in the quantity of gonad at the expense of perivisceral fluid has little effect on the total wet weight of the urchin.[4]

If the rate of respiration for unit wet weight does not increase with increase in the bulk of the gonads and the corresponding increase in organic content of the specimens, then the average rate of respiration per unit of organic matter in the animal must decline as the animal becomes more gravid. This is borne out when the respiratory rate is expressed in terms of nitrogen content in Figure 1B; as the gonad index increases, Q_{O_2} per unit nitrogen declines. This means that either the tissue in the gonad (or other tissues) becomes metabolically relatively less active, or the oxygen supply becomes limiting as the gonads grow. The respiration of the sea urchin body components was therefore investigated in order to determine which of these factors is involved in the reduction of Q_{O_2} during gonad growth.

2. *The respiratory rate of sea urchin body components*

The rate of respiration was determined for each of the sea urchin body components to ascertain the contribution of each component to the respiration of individuals 15 to 26 grams wet weight.

From data for the average respiration of sea urchin body components in Table I, it is evident that the Q_{O_2} for the perivisceral fluid is negligible. The Q_{O_2} for the lantern is low; the Q_{O_2} for ovary and body wall, intermediate; and the Q_{O_2} for testis and gut, very high. The rate of respiration of a body component appears to be similar from animal to animal, the data being fairly consistent as indicated by the standard deviations, except for the gut and testes. Part of the variability in mature testis Q_{O_2} may result from the liberation of sperm, activating them to different degrees, although the respiratory rate of testes with active sperm was not always high. It therefore seemed best to consider the data for testes in two categories: those with a high rate of respiration (A in Table I) and those with a low rate of respiration (B in Table I).

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[3] The perivisceral fluid values for sea urchins of gonad indices 1.0–3.2, 3.3–5.5, 5.6–7.0, 7.1–11.2 and 11.3–14.9 are 30.4 ±1.0, 25.5 ±2.4, 25.8 ± 4.3, 23.2 ± 3.5 and 21.6 ± 3.3, respectively.

[4] The other variable determining the amount of perivisceral fluid in an urchin is the amount of food stored in the intestinal festoons of the animal.

From the product of the Q_{O_2} for each of the body components and the component index $\frac{(\text{component wet weight} \times 100)}{\text{body wet weight}}$ it is possible to arrive at the theoretical oxygen consumption for each component constituting the sea urchin body. For a sea urchin 15–26 grams wet weight the body wall constitutes about 57.8% of the mass, the lantern 4.8%, the perivisceral fluid 26.8% (18.7% to 34.2%), the gut 4.6% and the gonads (depending upon the phase in the reproductive cycle) from 1.4% to 19.0% in the female and from 1.2% to 21.7% in the male. The product of each component index and the Q_{O_2} for the component is given in Table

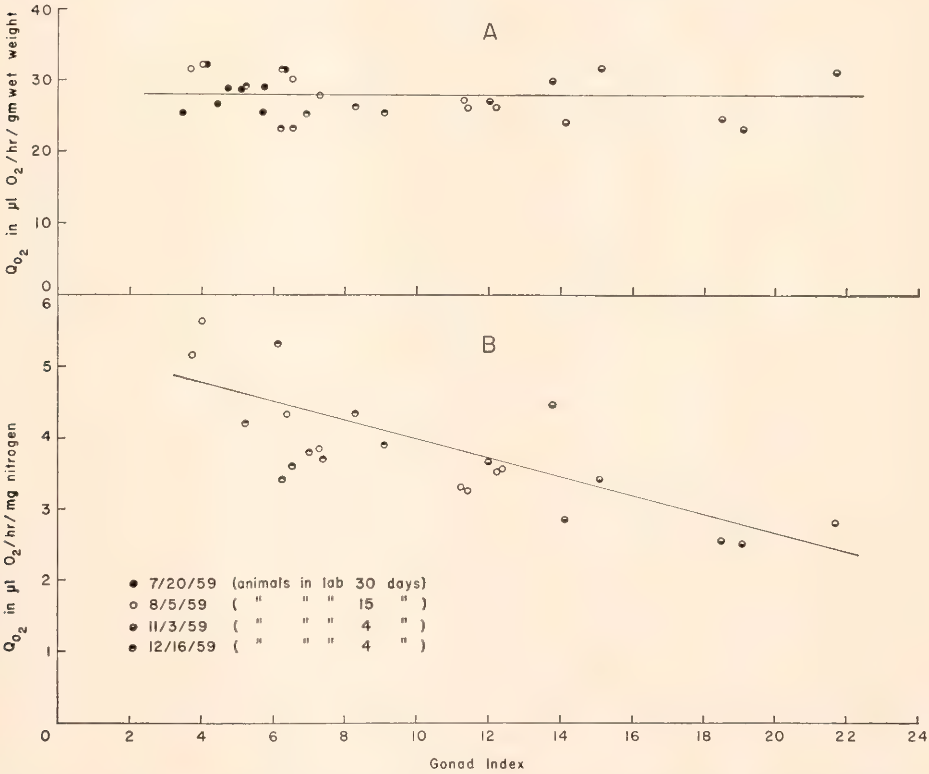

FIGURE 1A. Oxygen consumption of sea urchins in µl. O_2/hour/gram wet weight in relation to gonad index. A second group of data on 46 small sea urchins gathered during the 1964–65 season gave essentially the same results as the data cited here. These data covered a gonad index range of 0.5 to 15 for males and 1.5 to 8.5 for females.

FIGURE 1B. Oxygen consumption of sea urchins in µl. O_2/hour/mg. nitrogen in relation to gonad index. Experiments in A and B were done at the Hopkins Marine Station.

II (Column A × B). The ratio of each product to the sum of the products of the body components times 100 $\left(\frac{A \times B \times 100}{\Sigma A \times B}\right)$ gives the fraction of the theoretical respiration attributable to a given component (column 7). Both values for testis (see Table I) were used in these calculations for reasons cited above.

From the data in Table II it is evident that in the non-gravid sea urchin the body wall probably accounts for most of the respiration (72.7% in a female of gonad index 1.0 to 71.9% in a male of gonad index 1.0). In these sea urchins the gonad could account for only a small fraction of the respiration (1.6% of the respiration in the female and about 2.7% of the respiration of the male). When the gonad index reaches 20 at the height of the breeding season the fraction of respiration attributable to the gonads increases. The increase in gonad tissue is then expected to increase the respiration of the whole animal by 30% in the case of females, and 51% in the case of males (Table II, last column; if the high value for testis respiration is used (Table IA), the increase for the male is much greater: 124%). This expectation is reinforced by the finding that there was no consistent decrease in the oxygen demand of any tissue measured *in vitro* as the urchin became gravid. Thus the body wall and gut from males of gonad index 3–5.9 and 18–20.9 had Q_{O_2} values of 24.4 and 26.6, and 73.1 and 70.7, respectively. Testes and ovaries from urchins of gonad index 3–5.9 and 12–14.9 had Q_{O_2} values of 59.4 and 54.0, and 27.6 and 35.9, respectively.[5] Therefore, the expected increase in the oxygen uptake of the whole animal as it becomes gravid is not compensated for by reduction in the oxygen demand of any tissue and yet Figure 1A shows that the expected increase in fact does not occur.

TABLE I

Oxygen consumption (Q_{O_2} in µl. O_2/hour/gram wet weight) of body components in sea urchins 15–20 grams wet weight

Body component (B.C.)	B.C. index*	Q_{O_2}**	Number of experiments
Body wall	57.8 ± 7.5	28.3 ± 6.5	38
Lantern	4.8 ± 0.7	13.8 ± 4.7	17
Perivisceral fluid	26.8(18.7–34.2)	1.1 ± 0.8	9
Gut	4.6 ± .8	102.8 ± 33.3	35
Ovary	4.4(1.4–8.2)	36.6 ± 4.4	10
Testis A		145.0 ± 13.9	5
B	6.0(1.2–11.3)	62.1 ± 16.2	19

* The values following the ± sign are standard deviations

** For testis A values are for experiments with very high Q_{O_2}, B values for considerably lower Q_{O_2} (see text).

Direct comparisons were made of total oxygen consumption of an intact sea urchin measured in a large respirometric vessel and subsequently, the total oxygen consumption as determined by summation of the oxygen consumption of its dissected body components. In sea urchins of low gonad index the difference between the oxygen consumption measured in these two ways is usually not great but becomes striking, particularly for males of high gonad index. A total of 21 sea urchins were studied in this manner. The average respiration determined by summation of respiration of body components was greater than the respiration measured on the whole animal, verifying the assumptions in Table II. The theoretical respiration determined by summation of the respiration of body components of gravid urchins was usually greater than that calculated in Table II, especially in males. Thus the theoretical respiration for a male of gonad index 7.0 was 103% greater than that directly determined on the whole urchin, and for a male of gonad index 11.2 it was 179% greater. However, the data in Table II were caculated from average values for many measurements on each of the body components.

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[5] Sea urchins larger than 20 grams had to be included in these samples to get a sufficient span of gonad index.

TABLE II

*Oxygen consumption (Q_{O_2} in µl. O_2/hour/gram wet weight) of body components and the theoretical respiration of the entire 20-gram sea urchin calculated from these data**

Sex and state of urchin	Body component	A Component index	B Q_{O_2}	(A×B)	Σ(A×B)	% of total respiration $\frac{A \times B}{\Sigma(A \times B)} \times 100$	Theoretical respiration (A×B) for 20-gram urchin	$\frac{Q_{O_2} \text{ gravid} \times 100}{Q_{O_2} \text{ non-gravid}}$
♀ low G I**	Body wall	57.8	28.3	1635.7		72.7		
	Lantern	4.8	13.8	66.2		2.9		
	Fluid***	31.8	1.1	35.0		1.6		
	Gut	4.6	102.8	472.9		21.0		
	Gonad	1.0	36.6	36.6	2246.4	1.6	449.3	
♀ high G I	Body wall	57.8	28.3	1635.7		55.9		
	Lantern	4.8	13.8	66.2		2.3		
	Fluid	12.8	1.1	14.1		0.5		
	Gut	4.6	102.8	472.9		16.1		
	Gonad	20.0	36.6	732.0	2920.9	25.0	584.2	130
♂ low G I	Body wall	57.8	28.3	1635.7		71.9		
	Lantern	4.8	13.8	66.2		2.9		
	Fluid	31.8	1.1	35.0		1.6		
	Gut	4.6	102.8	472.9		20.8		
	Gonad	1.0	62.1	62.0	2271.8	2.7	454.4	
♂ high G I low testis Q_{O_2}	Body wall	57.8	28.3	1635.7		47.7		
	Lantern	4.8	13.8	66.2		1.9		
	Fluid	12.8	1.1	14.1		0.4		
	Gut	4.6	102.8	472.9		13.8		
	Gonad	20.0	62.1	1240.0	3428.9	36.2	685.8	151
♂ high G I high testis Q_{O_2}	Body wall	57.8	28.3	1635.7		32.1		
	Lantern	4.8	13.8	66.2		1.3		
	Fluid	12.8	1.1	14.1		0.3		
	Gut	4.6	102.8	472.9		9.3		
	Gonad	20.0	145.0	2900.0	5088.9	57.0	1017.7	224

* The Q_{O_2} data for body components are taken from Table I.

** G I refers to gonad index.

*** Perivisceral fluid.

There exists a possibility that the total oxygen consumption of a whole gravid sea urchin is less than that of its components measured separately because of factors other than lack of oxygen. Thus, tissue cells need not respire at the same rate inside the sea urchin as when they are removed, a case in point being sperm activated to swimming resulting in oxygen consumption higher than inside the testis of the urchin, although the respiratory rate was not high in all cases in which actively swimming sperm were seen. Possibly endocrine or other regulatory agents, lacking in dissected components, may determine the rate of respiration of the tissues inside an urchin although no evidence exists for such factors. However, the total respiration of the rat, which has a well-developed endocrine system, is only slightly more than the sum of the respiration of its parts (Field *et al.,* 1939). The total respiration of an intact snail which also has endocrines may be equal to the sum of the respiration of its parts, although usually it is less (Kerkut and Laverack, 1957). It seems more likely, however, that availability of oxygen to tissues is probably the major factor limiting respiration of tissues in the intact sea urchin. Oxygen tension of perivisceral fluids in several species of echinoderms tested was much lower than prevails in the bathing sea water; no such tests have yet been made on the purple sea urchin (see review in Farmanfarmaian, 1966).

DISCUSSION

The data reported in this paper demonstrate that there is no increase in respiration (Q_{O_2}) of the purple sea urchin corresponding to the increase in tissue during growth of the gonad from resting to gravid state. The data suggest that the expected augmentation in the total oxygen uptake of the gravid animal fails to occur because of insufficiency of the oxygen supply. The limits of oxygen supply are set by the ambient oxygen tension, the respiratory surface, and oxygen transport to the internal organs. Since the oxygen tension of the water containing the sea urchins was uniform in all the experiments, this parameter may be set aside from the present discussion. The respiratory surface and oxygen transport to the internal organs, however, are important matters for consideration. The test surface and the external appendages take up their oxygen from the ambient waters directly, and therefore there is no oxygen attenuation. The internal organs, on the other hand, receive their oxygen supply mainly through the ambulacral podia and ampullae. Within the coelom dissolved oxygen is transported by ciliary currents and other convection transport (Farmanfarmaian, 1959).

The haemal system, which may play a role in distribution of nutrients (Boolootian and Campbell, 1964), is unlikely to participate in distribution of oxygen because there is no way for oxygen to enter it except through the perivisceral fluid, and no respiratory pigment is present. Thus, the internal supply of oxygen is limited by the respiratory surface and the internal transport. When the sea urchin becomes gravid and the gonad tissue increases by 10- to 20-fold (and the nitrogen content of the sea urchin doubles), there is no commensurate increase either in the respiratory surface or in the internal oxygen transport. This presumably results in oxygen attenuation in the perivisceral fluid and relative oxygen unsaturation of tissues which are far from the respiratory surface (see review in Farmanfarmaian, 1966). However, no measurements of the oxygen tension of the perivisceral fluid of the purple sea urchin have yet been made. Oxygen unsaturation has, however, been demonstrated indirectly in echinoderms, including the purple sea urchin, as well as in other animals which, within limits, "conform" to the oxygen tension of their atmosphere. Asteroids such as *Patiria miniata, Asterias rubens,* and *Asterias forbesi* increase or reduce oxygen consumption in proportion to the ambient tension within a definite range (Hyman, 1929; Koller and Meyer, 1933; Meyer, 1935; Maloeuf, 1937). Such is said to be true for the purple sea urchin as well (Hyman, 1929, p. 532). Nomura (1926) reported that in the range of 0.72–5.7 cc. O_2/l., the rate of oxygen consumption in the sea cucumber *Paracaudina chilensis* is directly proportional to the oxygen tension of the medium. When these or other "conforming" organisms are minced, the oxygen consumption of the minced tissue is independent of the oxygen tension and is constant (Harnisch, 1932; Meyer, 1935). In short, the limitations of the respiratory surface and internal transport are eliminated by the mincing process; there is then no oxygen attenuation or oxygen unsaturation of the internal organs. Increase in ambient oxygen tension, therefore, does not then change the rate of oxygen consumption.

The respiratory rate of echinoderms increases with increase in environmental temperature, reaching a maximum between 26 and 30° C. in various animals (Farmanfarmaian and Giese, 1963; Farmanfarmaian, 1966). In the case of *S. purpuratus*, the respiratory rate at 20° C. is double that at 10° C. (not allowing time for acclimation). Under these conditions, while the metabolic rate increases, the respiratory surface and the ambient oxygen tension do not change (although the amount of oxygen dissolved in the sea water decreases). The increase in oxygen consumption observed at the higher temperature may be attributed to increased direct oxygen uptake by the external tissues (chiefly the body wall, which in the size range studied, makes up more than $\frac{1}{2}$ of the urchin bulk) and increase in ciliary convection transport of oxygen to the internal organs, allowing increased respiration of at least the surfaces of the organs if not of the deeper cells.

Finally, the discrepancy between Figures 1A and 1B becomes clear on the basis of the following considerations: Within a given reproductive cycle there is no significant change in the total wet weight of the animal because changes in amount of perivisceral fluid generally compensate for changes in gonad weight. Total respiration appears to be limited by the oxygen supply which is nearly constant throughout the reproductive season. The respiratory rate expressed in terms of wet weight, therefore, does not change significantly within a reproductive cycle (Fig. 1A). On the other hand, gram for gram, the total nitrogen of the gonad is much higher than that of the perivisceral fluid. There is a real and substantial increase in the total nitrogen of the animal, due to the growth of the gonads within a reproductive season. The respiratory rate expressed in terms of total nitrogen decreases as the gonads grow, although total respiration remains nearly constant throughout a reproductive cycle (Fig. 1B).

SUMMARY

1. The oxygen consumption of small sea urchins differing in gonad index, studied by Warburg-Barcroft manometry, is 26 to 31 µl. 0_2 per hour per g. wet weight, or between 3.0 to 5.6 µl. O_2 per hour per mg. nitrogen.

2. The rate of oxygen consumption per unit wet weight is approximately the same regardless of gonad index of the animals.

3. The gonad index measures the size of gonad relative to the entire body weight. In a sea urchin the wet weight does not change appreciably within a reproductive cycle as the gonad index increases because the perivisceral fluid, of much the same specific gravity as the tissues, is displaced by the gonads.

4. With increase in gonad index, however, the bulk of organic material as measured by nitrogen content in a sea urchin increases, ultimately doubling. Consequently, when the rate of respiration is measured per unit nitrogen content, it is found to fall with rise in gonad index.

5. When gonads and other body components are removed from the body, and oxygen consumption measured in sea water in equilibrium with air, their rate of respiration is approximately the same regardless of size or gonad index of the animal from which they came. When the gonad increases in size, the expected total respiration of the sea urchin, as determined by summing the respiration of its body components, is therefore greater than the respiration measured on the intact animal in water in equilibrium with air.

6. The rate of respiration of sea urchins is probably limited by the respiratory surface and convection transport of oxygen to the internal organs. Increase in the bulk of internal tissue due to gonad growth does not result in increased total respiration by the animal. Presumably, instead, coelomic oxygen tension becomes attenuated, resulting in the relative oxygen unsaturation of the internal tissues.

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PHALACROCLEPTES VERRUCIFORMIS GEN. NOV., SP. NOV., AN UNCILIATED CILIATE FROM THE SABELLID POLYCHAETE SCHIZOBRANCHIA INSIGNIS BUSH

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Of the known ciliates, two separate groups are characterized by the absence of cilia during at least a part of the life history. In the suctorians, the tentaculate and usually predaceous adults are unciliated, but typically they produce one or several ciliated larvae. The larvae, after a short free-living existence, lose their cilia and metamorphose into adults. Scattered argentophilic bodies found in at least some suctorians are thought to be persistent kinetosomes. Studies on the morphogenetic events involved in the production of larvae have shown that kinetosomes proliferate in local areas, then become organized in rows, forming the ciliary field of the prospective larva. In species of *Allantosoma,* which inhabit the digestive tract of horses, no ciliated stage has been reported. Although these organisms become attached to ciliates and probably feed in the same general way as suctorians, they may have evolved along entirely different lines.

Another group of unciliated ciliates belongs to the family Sphenophryidae, referred to the so-called Thigmotricha.[1] The sphenophryids are more or less sessile parasites of the ctenidia of lamellibranch molluscs. The kinetosomes of these bizarre holotrichs are arranged in definite rows, but only one of the several known species (*Lwoffia cilifera* Kozloff) is ciliated throughout its life history. In *Sphenophrya, Gargarius,* and *Pelecyophrya,* portions of the rows of kinetosomes found in the adult are conferred upon the larva, and cilia appear on these portions. The larva is quite similar to certain representatives of the family Ancistrocomidae, and it is presumed that ancistrocomids are ancestral to the sphenophryids.

In the course of studies on the biology of *Ignotocoma sabellarum* Kozloff (1961), an ancistrocomid ciliate living on the sabellid polychaete *Schizobranchia insignis* Bush, I found an entirely different kind of parasite on the pinnules of the prostomial cirri. This organism had no cilia, and its appearance while alive did not provide any good clues to its systematic position. After fixing and staining some specimens, however, I observed that these possessed both a macronucleus and a micronucleus, and that the nuclear events involved in reproduction by fission were like those characteristic of ciliates. Eventually, the occurrence of conjugation was established. There can be no doubt that this organism is an unciliated ciliate. It is perhaps a suctorian or a highly-specialized thigmotrich.

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[1] The thigmotrichs, as understood by authors who have attempted to survey the important groups of ciliates, probably do not constitute a completely monophyletic division. Moreover, some of the less highly specialized thigmotrichs are difficult to separate by sharp lines from hymenostomes and peritrichs.

METHODS

The ciliates were studied extensively in the living condition, with the aid of both bright field and phase contrast optical systems. Most of the morphological and cytological studies were based on specimens in smears of the prostomial cirri of *S. insignis* prepared on coverglasses. The fixatives used were those of Bouin, Duboscq and Brasil, Hollande, Schaudinn, Champy, and Flemming (the stronger mixture), as well as a saturated aqueous solution of mercuric chloride containing 5% of acetic acid.

For staining the smears, I tried a variety of methods with material fixed in each of the mixtures listed above. Experimentation was necessary because the micronucleus of the parasite is difficult to stain sharply, especially during certain phases of the process of conjugation. Azocarmine G, acid fuchsin, basic fuchsin (used according to Dippell and Chao's modification of De Lamater's method), iron hematoxylin, alum hematoxylin (progressive staining with a dilute solution), and the Feulgen method all stained the macronucleus intensely, and usually differentiated the micronucleus well enough so that it could be distinguished. As a rule, however, the micronucleus was most suitably demonstrated by iron hematoxylin or the Feulgen nucleal reaction, after fixation in Bouin's fluid, Duboscq and Brasil's fluid, or the mercuric chloride-acetic acid mixture. When the Feulgen method was used, hydrolysis in normal hydrochloric acid at 60° C. was allowed to proceed for 12 to 15 minutes.

Impregnation with silver albumose (Protargol) was tried after fixation in almost all of the fixatives used before staining procedures. The results did not vary a great deal so far as demonstration of certain argentophilic granules was concerned, although cleaner preparations were generally obtained after fixation in the mixtures of Bouin, Duboscq and Brasil, and Hollande. The Chatton and Lwoff method of impregnation with silver nitrate, following brief fixation in Champy's fluid and subsequent treatment with Da Fano's fluid, yielded preparations showing the same granules demonstrated by the Protargol method, but provided no additional information.

For studying the parasites *in situ* on the pinnules, material fixed in Bouin's fluid and embedded in paraffin was sectioned at 5 μ, and the sections were stained with iron hematoxylin. However, the shrinkage of the ciliates and the pinnules was so marked that the relationship of the parasites to the epithelium could not be demonstrated acceptably. Considerably more instructive preparations were obtained by fixing heavily-infected pinnules in osmium tetroxide buffered to pH 7.5 with *s*-collidine (0.5 ml. of *s*-collidine to 1 ml. of 4% osmium tetroxide), embedding them in Epon epoxy resin according to the procedure of Luft (1961), and cutting sections at 0.5 μ and 1 μ with glass knives.[2] The sections were stained by the methylene blue-azure II mixture recommended by Richardson, Jarett and Finke (1960). Shrinkage appeared to be negligible, and I hope that the relationship of the ciliates to the tissue was preserved rather faithfully.

Neutral red was used as a supravital stain for cytoplasmic granules, and lipid inclusions in fresh preparations were stained with Sudan IV in 50% alcohol.

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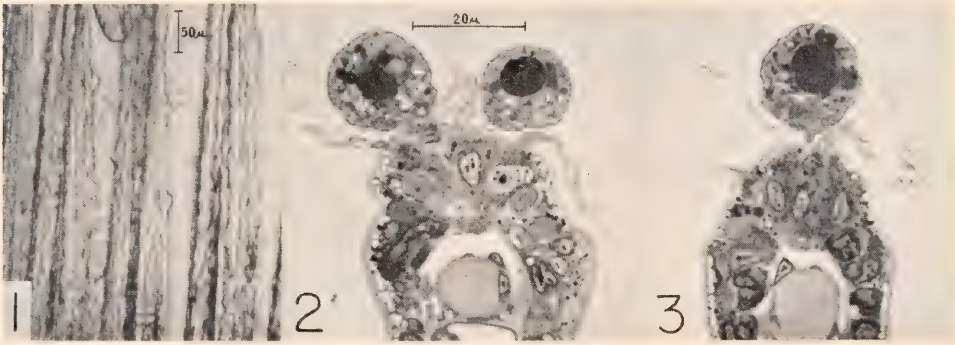
[2] Mr. John Boykin very kindly instructed me at certain points during the procedures of embedding and sectioning. His help is warmly appreciated.

Unsuccessful attempts to demonstrate glycogen were made with Lugol's iodine solution applied to fresh smears, and by fixation in a mixture of nine parts of absolute alcohol to one part of formalin followed by staining by Bauer's method.

Phalacrocleptes verruciformis gen. nov., sp. nov.

This interesting organism was found on *Schizobranchia insignis* collected on floating docks at two localities on San Juan Island, Washington. Of 115 specimens taken at Mitchell Bay (Long. 123° 10′ W.; Lat. 48° 34.2′ N.) and examined at various times within 14 days after the date of collection, 22 were observed to have at least a few ciliates. Of 140 worms collected at Roche Harbor (Long. 123° 05′ W.; Lat. 48° 36.6′ N.) and examined within 24 days after collection, 26 were definitely parasitized. However, as the parasites are sometimes very rare, I could not certify that a particular specimen of *S. insignis* was completely free of the ciliate unless I examined every one of the hundreds of pinnules of the prostomial cirri. All worms from both of the localities were parasitized by *Ignotocoma sabellarum.*



Explanation of Figures 1–3

Phalacrocleptes verruciformis. Photomicrographs.

Figure 1. Living specimens on pinnules of prostomial cirri of *S. insignis.*

Figures 2, 3. Transverse sections of specimens attached to pinnules. Buffered osmium tetroxide; Epon sections, 0.5 μ; methylene blue-azure II.

When cirri of a host sabellid are examined under lower magnifications, the ciliates appear as rather clear, wart-like protuberances (Figs. 1, 4). They are restricted to the frontal surfaces of the pinnules. *Ignotocoma sabellarum,* on the other hand, lives attached to the main stems of the cirri and their branches as well as to the pinnules, but it is not common on the frontal surfaces.

In most individuals of *S. insignis* observed to be parasitized by *P. verruciformis,* relatively few ciliates were present. Sometimes I noted only one or two specimens on widely scattered pinnules. In other instances, a number of specimens were found on several adjacent pinnules of certain cirri. When heavy and more or less general infestations were observed, perhaps half of the many pinnules on each cirrus had at least one or two parasites, and up to 12 specimens were observed on some pinnules. As a rule, the ciliates were more abundant on the pinnules arising from the distal half of a particular cirrus than from the proximal half; in certain cases, however, the reverse was true, and in many hosts the parasites were rather evenly distributed.

The contact of the ciliates with the pinnules is evidently rather tight. When entire pinnules are dropped into a fixative, the ciliates rarely fall off; in fresh preparations, they remain attached until they are moribund. Even when smears are prepared, some of the parasites may remain *in situ*.

The shape of *P. verruciformis* varies considerably. When several living specimens from a single pinnule or from different pinnules are compared, no two of them may be quite alike. However, although the ciliates are capable of slow changes in general shape or in the shape of certain portions of the body, I have never observed specimens to move actively from one part of a pinnule to another.

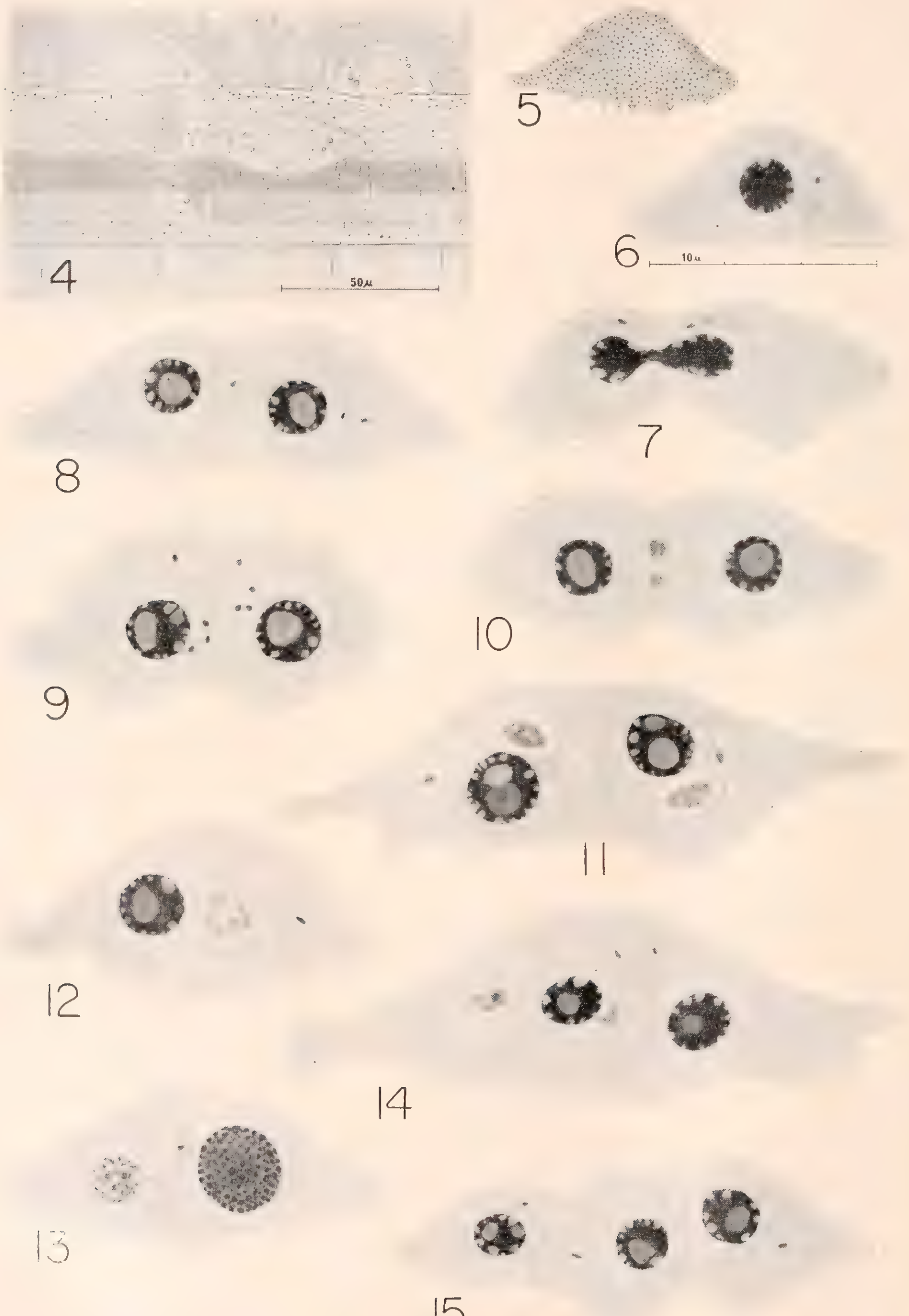
When viewed from the side (Fig. 4), the outline of the body ranges from one which is semicircular to one which is humped in the center but less convex near the extremities. The size (length × thickness) of specimens observed from this aspect ranges from about $27 \times 13\ \mu$ to about $50 \times 17\ \mu$. When the ciliates are viewed with the superior or inferior surface uppermost, the outline varies from broadly oval to fusiform; the width is one-third to two-thirds the length.

The character of the inferior surface is perhaps the most variable feature of this ciliate. In some specimens, it is so markedly flattened that the body is approximately semicircular in transverse section; in others it is more or less rounded (Fig. 2) or ridge-like (Fig. 3) in transverse section. The plasticity of the inferior surface is apparent not only in gross changes of shape which it may undergo, but also in the production, withdrawal, and alteration of protuberances and marginal sinuosities. Portions of the body which are not close to the epithelium of the pinnule are sometimes slightly wrinkled, but lack definite protuberances.

The inferior surface also characteristically exhibits a number of very delicate cylindrical or conical projections, which are probably used for obtaining food from the epithelial cells of the pinnule. These projections are difficult to see in living specimens, probably because they tend to be withdrawn. Evidently they are not well preserved by most of the fixatives I tried on smear preparations; Champy's fluid and Flemming's fluid were the only ones which gave moderately acceptable results as far as these projections were concerned. However, in specimens impregnated with Protargol after fixation in the mixtures of Bouin, Hollande, and Duboscq and Brasil, they are often very clear, even if obviously shrunken (Fig. 5). They show up best in material in which the impregnation is at least moderately heavy. The projections are quite evident in thin Epon sections of ciliates fixed *in situ*. They can definitely be seen to contact the surfaces of epithelial cells, but I have never seen them penetrating the cells. The pellicle surrounding these projections, especially at their distal portions, appears to be thinner than that surrounding the body of the ciliate as a whole.

The cytoplasm of living specimens contains abundant small granules up to about $0.7\ \mu$ in diameter. These are usually more or less evenly distributed, but sometimes they are concentrated in clusters; they exhibit no obvious Brownian movement. The granules are refractile and very distinct when examined with a phase contrast system, and they are stained deep red by neutral red.

In smear preparations which have been fixed and stained, the granules which


4
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Figs. 4–15.

are conspicuous in living ciliates are not often evident. They are visible in thin Epon sections stained by methylene blue and azure II, but are not very distinct because the cytoplasm is usually stained almost as deeply. Protargol impregnates a large number of small cytoplasmic granules (Fig. 5), and those which are close to the pellicle appear much like kinetosomes. What seem to be the same granules are often distinctly blackened by the Chatton and Lwoff method of impregnation with silver nitrate. At no time during fission or conjugation do the granules near the surface of the body form any particular pattern or show a tendency to become more heavily concentrated in certain regions; they simply remain more or less evenly distributed. In any case, it seems unlikely that any of them can be homologized with kinetosomes.

The macronucleus is evident in living ciliates as a spherical and almost homogeneous body. None of the fixatives which I used for smear preparations preserved the macronucleus very well. Most of them caused the chromatin to be clumped in coarse masses (Fig. 6); the numerous small vacuoles commonly noted in peripheral areas of the macronucleus in fixed specimens, especially those fixed in the mercuric chloride-acetic acid mixture, appear to be artifacts. In thin sections of material fixed in buffered osmium tetroxide and embedded in Epon (Figs. 2, 3), the macronucleus shows what appears to be a more natural form: a smooth outline and nearly homogeneous contents.

The small micronucleus is usually ellipsoidal or fusiform. It is rarely distinguishable in living specimens, even when a phase contrast optical system is used. The position of the micronucleus varies, but it is more likely to be found in the upper half of the ciliate. As has been pointed out previously, it is not often stained intensely by any method I have tried.

As far as I have been able to determine, there is no contractile vacuole. No inclusions which can be identified as constituents of the epithelial cells of the host have been recognized within the cytoplasm. There are, however, a number of scattered lipid inclusions; the largest of these reach a diameter of about 3.5 μ. In thin Epon sections (Figs. 2, 3), there are characteristically a number of masses

EXPLANATION OF FIGURES 4–15

Phalacrocleptes verruciformis. All figures except Figure 4 were prepared with the aid of a camera lucida to the scale accompanying Figure 6, and unless otherwise noted are based on specimens fixed in mercuric chloride-acetic acid and stained by the Feulgen nucleal reaction and orange G. (The character of the macronuclei is not well preserved.)

FIGURE 4. Living specimens on frontal surface of pinnule of *S. insignis.*

FIGURE 5. Projections of inferior surface, and argentophilic granules. Bouin's fixative; Protargol.

FIGURE 6. Typical trophic individual.

FIGURE 7. Binary fission.

FIGURE 8. Conjugation, prior to first division of micronuclei.

FIGURE 9. First two micronuclear divisions completed.

FIGURE 10. Synkaryons in region of contact of conjugants.

FIGURE 11. Old macronuclei, new macronuclei, and micronuclei in conjugants.

FIGURE 12. Exconjugant.

FIGURE 13. Exconjugant with old macronucleus undergoing resorption and large new macronucleus.

FIGURE 14. Individual with two old macronuclei, two new macronuclei, and two micronuclei; probably the result of complete fusion of conjugants.

FIGURE 15. Conjugation of three individuals.

within the cytoplasm which have a rather vague outline and which are stained deep blue by methylene blue-azure II. Most of these masses are close to the macronucleus, and some appear to be in actual contact with it. Their significance is not clear.

Binary fission in this ciliate follows the conventional pattern. Division of the micronucleus is completed as the macronucleus elongates. Constriction of the macronucleus into two more or less equal masses evidently takes place not long before separation of the daughter ciliates is completed (Fig. 7). Extrusion of macronuclear chromatin during division has not been observed.

Stages of conjugation are very abundant in my preparations. However, the micronuclei often stain so weakly that it is impossible for one to be sure that all of the micronuclei—including those undergoing resorption—have been accounted for. The behavior of the micronuclei is particularly difficult to follow at certain stages when they are almost Feulgen-negative. I have never been able to distinguish what I could be sure were chromosomes. My interpretation of the sequence of nuclear changes involved in what I believe to be typical conjugation may therefore not be completely correct.

When two specimens which are close together on the same side or opposite sides of the field of frontal cilia become joined in conjugation, they remain tightly affixed to the pinnule. The lobations of the margins of the inferior surface may persist; they are, in fact, very prominent in many conjugants. In fixed and stained preparations, the macronucleus of recently-united individuals (Fig. 8) characteristically exhibits at least one large vacuole-like clear area in addition to the numerous smaller peripheral vacuoles found in the macronuclei of non-conjugating individuals.

The micronucleus of each conjugant divides twice. Three of the four micronuclei produced by these divisions (Fig. 9) are resorbed. The other micronucleus divides to form the two gametic nuclei. This division typically takes place very close to the region of contact between the conjugants. The synkaryons seem actually to be formed between the conjugants (Fig. 10), and then to re-enter the cytoplasm; they are surprisingly poor in Feulgen-positive material.

I have never observed stages in the division of the synkaryon. However, it appears likely that the micronucleus and young macronucleus found in exconjugants (Fig. 12) and members of many conjugating pairs (Fig. 11) are ordinarily produced by just one division.

At first, much of the young macronucleus is occupied by an irregular, almost Feulgen-negative mass (Fig. 11). After the macronucleus reaches maximum size, the material peripheral to this mass gradually becomes richer in Feulgen-positive material. In time, large granules of very strongly Feulgen-positive chromatin fill the macronucleus (Fig. 13), and what was once a sizeable central mass appears to be represented only by one or more small remnants. In time, the young macronucleus becomes slightly smaller than its maximum size, and begins to resemble the macronucleus of trophic individuals.

The degeneration of the old macronucleus evidently takes place rather slowly. It often does not appear to change a great deal between the start of conjugation and the time the development of the new macronucleus has been almost completed. Eventually, however, the chromatin of the old macronucleus becomes severely disrupted (Fig. 13), and then very much condensed into a nearly homogeneous and intensely Feulgen-positive mass. A few small granules of chromatin are sometimes observed around the main mass of the degenerating macronucleus.

Certain stages which I have observed rather frequently do not fit into the pattern I have described. Some of them perhaps represent stages in alternate pathways of development of exconjugants, and others are probably anomalies. Individuals with two micronuclei, two old macronuclei, and two new macronuclei (Fig. 14) are moderately common. It seems likely that these result from complete fusion of conjugants. In some exconjugants, or in one or both of two conjugants which are still paired, there are two young macronuclei, one of which may be more advanced than the other. This suggests that the first micronuclear division following division of the synkaryon may also give rise to a macronucleus. The presence of two micronuclei and four young macronuclei in addition to two old macronuclei suggests that this has taken place in both of two conjugants which have fused completely. Conjugation between three individuals (Fig. 15) is observed occasionally, and I have also seen what is probably union of one individual with the product of a complete fusion of two others.

Three syntype slides of *P. verruciformis,* prepared from material collected at Mitchell Bay, San Juan Island, Washington, have been deposited in the United States National Museum (USNM Nos. 23751–23753).

The characteristics of the new genus *Phalacrocleptes* may be summarized as follows: Outline of the body, as viewed from one side, ranging from semicircular to slightly elongated and humped in the center; as viewed from the superior or inferior aspect, oval to fusiform. Inferior surface, in contact with the epithelium of the host, rounded, ridge-like, or somewhat flattened, generally with protuberances or marginal sinuosities; the character of the inferior surface and protuberances subject to considerable alteration. Delicate cylindrical or conical projections arising from the inferior surface contact the epithelium of the pinnules of the host and probably serve as feeding organelles. Macronucleus and micronucleus present. Cilia absent. Kinetosomes believed to be absent. No contractile vacuole. Nuclear changes during reproduction by binary fission proceeding as in typical ciliates. Conjugation in general similar to that of other ciliates. Type species: *Phalacrocleptes verruciformis* Kozloff, inhabiting the pinnules of the prostomial cirri of the sabellid polychaete *Schizobranchia insignis* Bush.

As this organism cannot at the present time be conscientiously assigned to an existing family, I propose it be referred to a new family, the Phalacrocleptidae. The distinguishing characteristics of this family are those of the type genus, *Phalacrocleptes.*

Discussion

Although the parasite which I have described apparently has no cilia at any time, and probably has no kinetosomes, the behavior of its nuclei during fission and conjugation clearly indicates that it is a ciliate. Finding a lower taxon into which it can be fitted with some conviction is more difficult.

Perhaps this curious organism is a suctorian which does not produce a ciliated larva. However, there is a remote possibility that it is a relative of the ancistrocomids parasitizing sabellids. Aside from the fact that it occurs on a type of polychaete known to be populated by representatives of this already quite highly specialized group, there is no other evidence to influence my speculations concerning its possible origin.

If *P. verruciformis* is derived from an ancistrocomid parasitizing sabellids, its superficial similarity to the unciliated sessile stage of *Sphenophrya, Pelecyophrya,* and *Gargarius* cannot very well be taken as an indication that it is closely related to the sphenophryids. All known sphenophryids are parasites of the ctenidia of pelecypods, and are probably derived from a group of ancistrocomids associated with these mollusks.

Summary

Phalacrocleptes verruciformis lives on the frontal surfaces of the prostomial pinnules of *Schizobranchia insignis* (Sabellidae). Delicate projections extended from its changeable inferior surface contact the epithelial cells of the host and probably serve as feeding organelles. Although it has no cilia at any stage in its life history, and probably no kinetosomes, *P. verruciformis* has both a macronucleus and micronucleus, and reproduces by binary fission in much the same way as most other ciliates; moreover, the sequence of nuclear changes involved in its conjugation fits the pattern for ciliates in general. The systematic position of this organism within the group of ciliates is uncertain. It is perhaps a suctorian which does not produce ciliated larvae. There is also a possibility, however, that it is related to the ancistrocomid thigmotrichs which parasitize the prostomial pinnules of sabellids.

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TIME AND INTENSITY OF SETTING OF THE OYSTER, CRASSOSTREA VIRGINICA, IN LONG ISLAND SOUND

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The literature on the propagation of oysters is extensive. It consists of hundreds of articles in numerous publications, many of which are obscure State and local reports of comparatively limited circulation. Articles on spawning and setting of the American or Eastern oyster, *Crassostrea virginica* Gmelin (until recently known as *Ostrea virginica*), constitute by far the larger portion of the literature dealing with this aspect of oyster biology.

It would be impossible to offer here a comprehensive review of this voluminous literature. Furthermore, such review is unnecessary because the majority of the articles on the propagation of oysters have been included by Baughman (1948) in his annotated bibliography. Korringa (1952) commented on many articles that appeared after Baughman's bibliography was published, and recently Galtsoff (1964) offered another comprehensive résumé. Thus, with the exception of a relatively few papers published during the last year or two, I shall mention principally the articles directly pertaining to the propagation of oysters in New England waters, especially in Long Island Sound, where most of the work reported in the present paper was done.

A general description of the Long Island Sound region has been given in several previous papers (Prytherch, 1929; Loosanoff and Engle, 1940; Loosanoff, 1964). It will therefore be sufficient merely to point out here that the oyster setting grounds in Long Island Sound are confined principally to the Connecticut side, where the outline of its shore is irregular, composed of numerous harbors, bays, and other indentations. Many streams and rivers enter the Sound on the Connecticut shore; some form extensive estuaries, as that of the Housatonic River.

Long Island Sound has been an extremely important area since the cultivation of oysters was begun in the United States. One of the best historical reviews of the Connecticut oyster fisheries was given by Sweet (1941). As early as 1845, seed oysters collected from natural beds were planted in other areas and, eventually, plantings of large quantities of seed oysters were made in deeper waters of Long Island Sound on grounds which normally did not produce these mollusks. The planting of old shells to catch oyster set was initiated along the Connecticut shore of Long Island Sound in 1847, 8 years before the famous French oyster expert, Coste, began his extensive and successful experiments on cultivation of oysters in France.

During the prosperous era of the Connecticut oyster industry, the cultivated beds extended in a strip three to five miles wide, from Stony Creek to Stamford, a distance of about 40 miles. At one time nearly 50,000 acres of bottom were under cultivation.

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According to the records of many Connecticut oyster companies, only a few of which still exist, and reports of the Connecticut Shellfish Commission, heavy oyster sets were routine during the last quarter of the 19th century (Loosanoff and Engle, 1940). Between 1904 and 1925, however, a period of 22 years, not a single heavy set occurred in the Sound. Since 1925, good sets have occurred in Long Island Sound in only a few years, namely, 1930, 1939, 1940, 1941, 1944, 1945, 1955, and 1958. The other years have been complete failures or so-called marginal years during which either the set was general but too light to be commercially important, or the set was good in some sections of the Sound but failed in the major portion of the oyster-producing area.

The method which Connecticut oyster farmers use to obtain new oyster generations consists of spreading on the oyster beds each summer large quantities of old shells to which metamorphosing larvae can attach. In some years, when the industry experienced periods of prosperity, over a million bushels of oyster shells were planted in areas where setting of oysters could be expected. Plantings extended from the mean low-water mark to a depth of 40 feet or sometimes deeper. When setting failed, the planting of shells resulted in heavy monetary losses.

Because of infrequent and generally light sets, the oyster industry began to feel the lack of seed oysters and looked for explanations for the failure of oysters to set in Connecticut waters. Furthermore, since the oystermen had no knowledge as to when the heaviest periods of setting might occur, they often planted shells either too early or too late to obtain the best sets, even during good years. This situation existed in the early 1920's, when the U. S. Bureau of Fisheries detailed an investigator to Connecticut to work on the problem of lack of set (Prytherch, 1929). The original and primary purpose of these studies was to determine, if possible, the presence of clear-cut relationships between successful propagation of oysters and the conditions of their environment and, on this basis, to develop methods for predicting well in advance the time and intensity of setting of oysters.

It was believed originally that not more than 5 to 7 years would be required to gather the necessary data. For several reasons, however, these investigations had to be continued over a much longer period; eventually they covered more than a quarter of a century. Within a few years after beginning these observations it was discovered that variations in time and intensity of oyster setting were not as simple to explain and predict as had been first thought (Prytherch, 1929). Moreover, in the early 1930's I began to issue special bulletins for the information of the oyster growers on gonad development, spawning and setting of oysters, and survival of oyster set. These bulletins became of considerable interest and value to the oystermen and they insisted on a continuation of this work. During these studies over 60 articles were published on various aspects of biology of oysters, several of which are referred to in this paper. Many others on improvement of methods of oyster cultivation and control of their enemies were also written. Some of the latter, for example, the observations on spawning and setting of the common starfish, *Asterias forbesi,* of Long Island Sound, have been published (Loosanoff, 1964).

All of the results of these investigations cannot be conveniently discussed in a relatively short paper. The present article, therefore, is devoted to a description of the chronological aspects of spawning and setting of oysters in Long Island Sound and is to be followed by two more on the numerous biological and ecological variables that affect the propagation of oysters. To be included in the later articles are statistical analyses of the data to evaluate their relative importance and their possible value in predicting some important aspects of spawning and setting.

The data in this article are based upon observations made at many stations, but chiefly those at a depth of 30 feet or less. Statistical analyses already reported (Loosanoff, 1964) have shown that conditions at stations located at 30 feet and less are representative for the entire Long Island Sound.

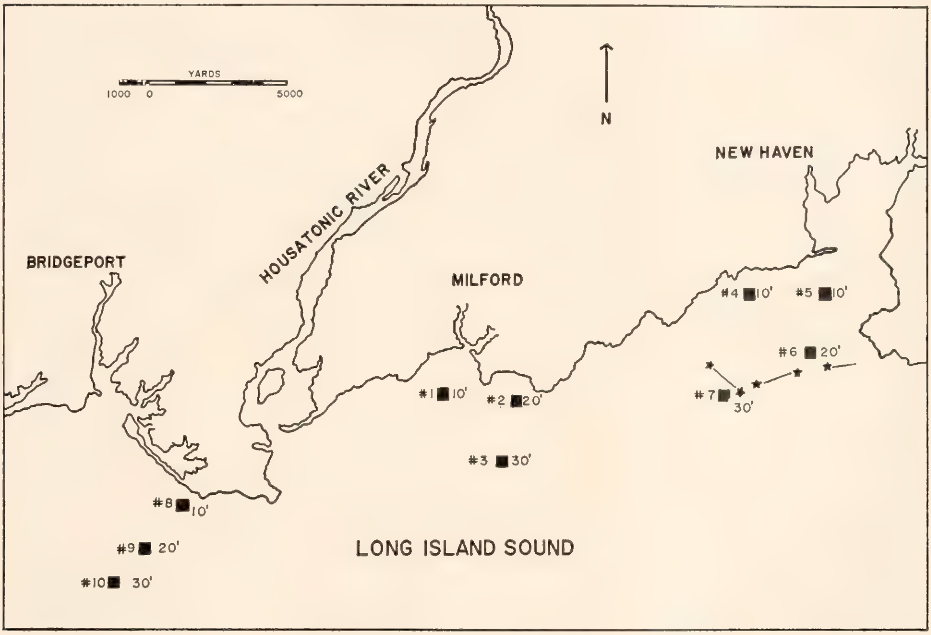

FIGURE 1. Location and depth of 10 stations established for observations on time and intensity of setting of oysters in Long Island Sound, 1944–61.

METHODS

These studies were conducted from 1937 to 1961 at the U. S. Bureau of Commercial Fisheries Biological Laboratory, Milford, Connecticut. In 1937–43 the chain of stations sometimes extended from Thimble Islands, east of Branford, to Westport (Loosanoff and Engle, 1940). In 1944–61 (except 1957, when only three stations were maintained), observations were carried on at the same 10 stations, located between New Haven and Bridgeport (Fig. 1). The studies dealt with various aspects of the propagation of oysters, including gonad development, discharge of spawn, occurrence and distribution of larvae, and the beginning and end of setting of larvae. The variations in time and intensity of setting throughout each summer constituted part of this investigation. Included also were systematic studies of environmental factors, among them changes in temperature, salinity, and pH of the water, amount of precipitation, river discharge, hours of sunshine, direction and velocity of winds.

The time and intensity of setting in different areas were determined by examining special collectors placed at each station. These devices (Fig. 2), which were wire bags filled with clean oyster shells, were first suggested in 1925 by Captain E. C. Wheeler of the Connecticut Oyster Farms Company. We have used this type of collector successfully in Virginia (Loosanoff, 1932), and later throughout the entire period of our studies in Connecticut.

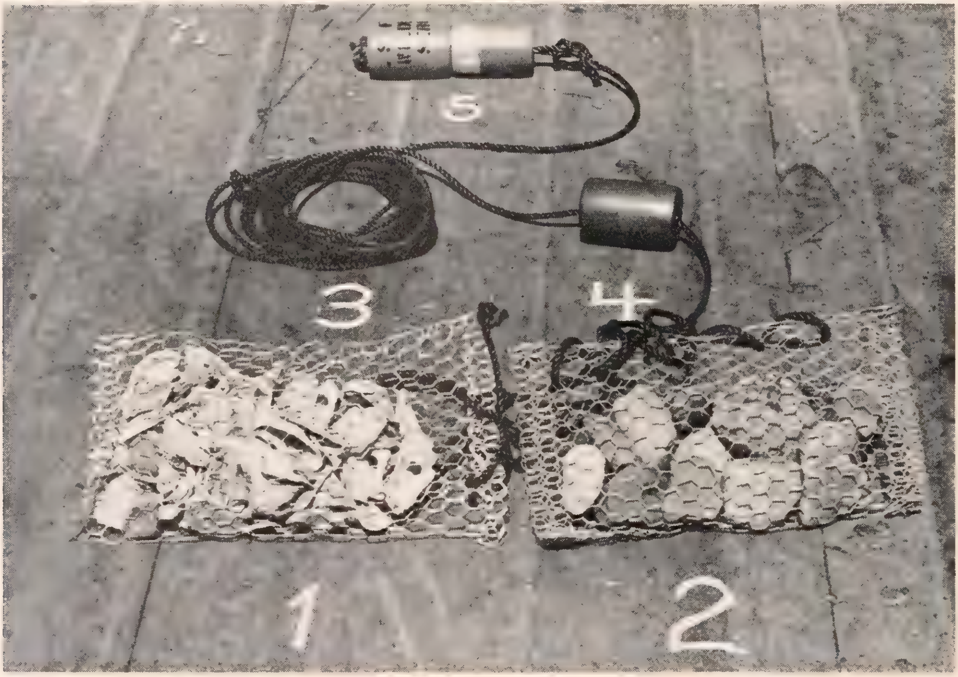

FIGURE 2. Type of collectors used for observations on setting of oysters in Long Island Sound. Description in text.

The wire-bag collector (Fig. 2-1) contained about 35 or 40 oyster shells of more or less uniform size. Two bags were used at each station. Another bag (Fig. 2-2), filled with small rocks, served as an anchor. A treated rope (Fig. 2-3) was run from the shell bags to the anchor bag, then to the auxiliary buoy (Fig. 2-4), which supported the middle portion of the rope, especially after it became waterlogged. A brightly colored buoy (Fig. 2-5) indicated the location of the collector.

Collectors were changed twice a week. The bags were brought into the laboratory and the shells examined within a few hours. The men counting the set were trained to distinguish oysters of different ages, from the time of attachment until they were about four days old. The set was counted on the inside surfaces of 20 shells taken at random from each collector and the results were later expressed as the number of oyster spat per 100 shell surfaces. This method is identical to the one used in studies of variations in time and intensity of starfish setting in Long Island Sound (Loosanoff, 1964).

Since two collecting bags were used to gather the data for each station and for each collection date, a pair of values was always available. It was possible, then, to correlate these values and to obtain an estimate of the reliability of the data. This analysis was made for two years, 1944 and 1955, which were chosen because the set of oysters was fairly heavy both years and because both years were characterized by considerable heterogeneity among the various stations. The only collection dates included in the analysis were those on which the set was appreciable, 10 or more oyster set in at least one of the 20 bags. For each date a correlation was computed between oyster set in bag A and oyster set in bag B from each of the 10 stations. These correlations ranged from 0.65 to 0.98 in 1944 and from 0.66 to 0.98 in 1955; the agreement between the two samples taken at the same time and place was excellent.

OBSERVATIONS

Systematic observations on gonad development and spawning of the oysters of Long Island Sound were conducted every summer from 1937 to 1956, a period of 20 years. A detailed description of gonad development has recently been given (Loosanoff, 1965).

In general, the spawning period in Long Island Sound waters lasts 2½ to 3 months; this time is considerably shorter than in warmer southern waters where, in some areas, spawning may continue throughout the greater part of the year (Hopkins, 1954). Prytherch (1929) offered a method which, in his opinion, was reliable enough to predict the start of spawning of oysters in Long Island Sound one month in advance. According to him a prediction could be made on the basis of several factors, of which water temperature from April to July was the most important. The strange part of Prytherch's proposal was the statement that the prediction could be made between July 1 and July 10 of each year, a period which obviously is too late for such a prediction because the spawning in Long Island Sound normally starts between June 26 and July 3 (Loosanoff, 1965).

Observations on occurrence and distribution of oyster larvae in the inshore waters of Long Island Sound constituted an integral part of this study. Unfortunately, they could not be carried out over as broad a scope as was desired, because of scarcity of personnel. Nevertheless, even these limited observations provided rich information of considerable biological interest.

Plankton samples for determination of the number of oyster larvae were collected by filtering known volumes of sea water through plankton nets. In our earlier studies, 50-gallon samples were pumped from near the surface and bottom, but because oyster larvae were rather uncommon, the volume of samples was later increased to 100 and eventually to 200 gallons. To facilitate the collection of such large samples the water was filtered through two nets: first, a coarser one made of #10 plankton netting, which retained most of the zooplankton, including larger bivalve larvae, and then through a #20, net, which retained the smaller larvae. The use of a #10 net prevented the clogging of the finer #20 mesh. This method is still used at Milford.

Several students of oyster propagation (Baughman, 1948) have made observations on the distribution of oyster larvae in Long Island Sound. In earlier articles, including Loosanoff and Engle (1940), many figures were based on the counts of younger, so-called straight-hinge stages of larvae. Since oyster larvae at that stage have an appearance almost identical to larvae of some other species, for example, those of *Mulinia lateralis,* these early counts were not wholly reliable.

Similar errors cannot be made, however, in identification of older oyster larvae, measuring 100 microns or more. The oyster larvae of this size are unique in appearance; no larvae of other bivalves of Long Island Sound can be mistaken for them, with the possible exception of larvae of *Teredo.* Even larvae of *Teredo* and oysters are sufficiently different to be distinguished by an experienced worker.

Since the studies on distribution and occurrence of oyster larvae covered more than 25 years, they are too extensive to be discussed in detail here. Only a brief summary of this subject is offered, therefore. In general, oyster larvae in Long Island Sound, especially those of advanced stages, were never numerous; they were unevenly distributed over the oyster-producing areas, and their appearance or disappearance did not follow any definite pattern.

Frequently the samples of larvae were composed entirely of individuals of advanced stage of approximately the same size. This observation was interpreted to mean that the larvae probably originated in an area other than the place they were collected and were brought along as advanced larvae with masses of water which retained their identity during the entire period of larval development.

Our efforts to correlate the numbers of larvae with some obvious factors of their environment were mostly unsuccessful. The literature contains several referances (including that of Nelson, 1925) in which unsuccessful setting of oysters is attributed to ctenophores that have, presumably, eaten the majority of the larvae originally present. During the studies we had many opportunities to test this theory. We found, however, that no clear-cut relation existed between the numbers of ctenophores and the failure of oyster set. For example, ctenophores were so numerous in the Bridgeport-New Haven area between July 28 and August 12, 1944, that swimming was discontinued at many beaches, and collection of plankton samples was practically impossible even though several straining devices were used to prevent the ctenophores from entering the nets. Yet, this period produced one of the heaviest oyster settings in the history of Connecticut oyster farming.

Neither could the numbers of larvae be correlated with the numbers of parent oysters living in Long Island Sound. For example, in 1937, when more than a million bushels of adult oysters were growing in the Sound, many plankton samples taken in the middle of summer contained no oyster larvae (Loosanoff and Engle, 1940). On the other hand, in 1958, when the adult oyster population was extremely small, oyster larvae, especially those in advanced stages, were numerous, sometimes as common as three mature larvae per gallon of water.

Dead or dying bivalve larvae were found in our samples during some summers. One such observation was made in 1959 at Stations 2 and 5 in New Haven Harbor. Samples collected on August 20 contained many living larvae that appeared abnormal. A few days later, on August 24 and especially August 31, many dead or dying larvae were taken.

Sufficient evidence is available to suggest that the absence or disappearance of the larvae from the waters of Long Island Sound may often be due to the plankton blooms that occur in this area in the summer. These blooms probably produce large quantities of external metabolites which are detrimental to larvae. It has been shown experimentally at our laboratory that a dense bloom of dinoflagellates causes abnormal development of eggs and larvae of the clam, *Mercenaria mercenaria,* and the oyster (Davis and Chanley, 1956). Even when eggs of oysters were placed in water from which the dinoflagellates were removed by a Millipore filter, the development of the eggs and larvae was still abnormal. It is also possible that during the periods of heavy blooms of dinoflagellates, such as *Prorocentrum triangulatum,* some nannoplanktonic forms, which constitute the food of oyster larvae, disappear and, as a result, the larvae may starve or grow slowly and be more susceptible to diseases.

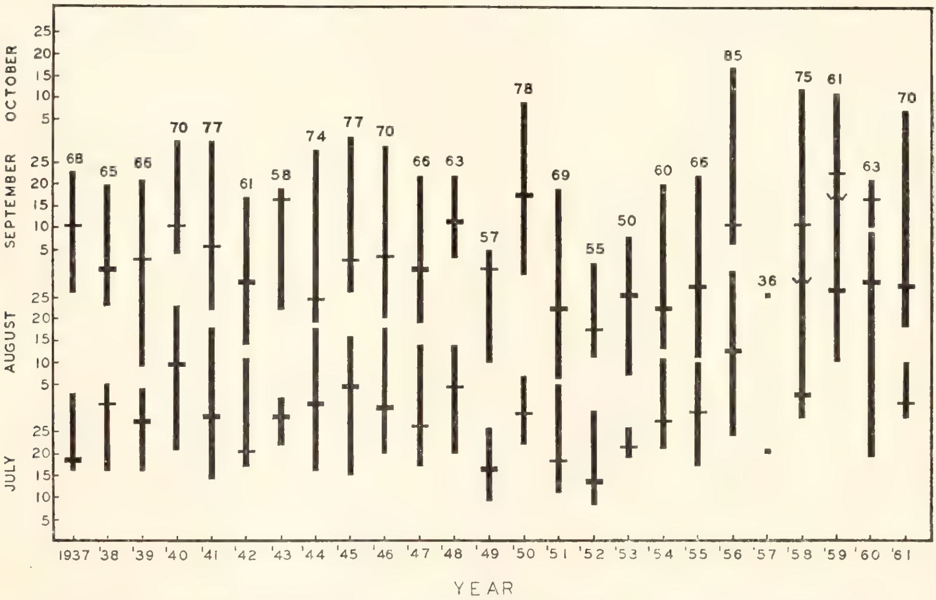

FIGURE 3. Beginning, end, and extent of setting period of oysters in different years in Long Island Sound, 1937–61. Cross-bars indicate the peaks of first and second waves of setting, the broader bars indicating the peaks of the heavier waves. Figures at top of each bar show number of days in setting period of each year.

The latest date at which mature larvae were found in our samples was October 8 (1959) when several were taken at Station 2, Milford Harbor, and 17 immature larvae were collected at Station 10 in Bridgeport Harbor.

In general, the length of the setting period of *C. virginica* increases from north to south. In Long Island Sound, as is shown later, this period extends from approximately the middle of July until the end of September or even early October.

TABLE I

Numbers of oyster set on 100 shell-surfaces, recorded semi-weekly at stations at 10 different depths off Stratford Point, Long Island Sound, 1939

Station number	1	2	3	4	5	6	7	8	9	10
Depth in feet	0	5	10	20	30	40	50	60	70	100
Date	Numbers of oyster set									
July 13	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0
20	120	20	20	60	60	0	10	0	0	0
24	590	1030	960	630	290	160	30	50	0	0
27	21560	26560	9980	13690	29980	9630	1420	370	20	10
31	6760	14340	27260	30430	21830	10040	1100	840	110	160
Aug. 3	310	570	440	310	140	0	40	20	10	0
7	0	0	0	0	0	0	0	0	0	0
10	0	0	0	0	0	0	0	0	0	0
14	0	0	0	0	0	0	0	0	0	0
17	0	0	0	0	0	0	0	0	0	0
21	10	10	0	10	0	0	0	0	0	0
24	0	0	0	0	10	0	0	0	0	0
28	0	50	30	10	30	0	30	0	0	0
Sept. 1	0	0	0	20	20	10	0	0	0	0
4	20	20	40	190	480	10	30	10	0	0
7	100	60	40	200	200	30	0	0	0	0
11	40	30	10	40	70	40	0	0	0	0
14	0	30	30	10	0	0	10	0	0	0
19	10	0	10	20	0	0	0	0	0	0
21	10	10	0	0	0	0	0	0	0	0
25	0	0	0	0	0	0	0	0	0	0
28	0	0	0	0	0	0	0	0	0	0
Station total	29530	42730	38820	45620	53120	19920	2670	1290	140	170

In Chesapeake Bay it normally continues from the middle of June until the middle of October (Loosanoff, 1932; Beaven, 1954). In South Carolina, however, the season is considerably longer than at either of the northern points just mentioned; it extends from early May through a large part of October (McNulty, 1953). Finally, according to Hopkins (1954) larvae of *C. virginica* set from April until November along the Gulf of Mexico coast. Obviously, the length of the setting period depends upon the temperature; it is longer where the climate is warmer.

During the 25 years of observations in Connecticut waters, the beginning of setting varied from year to year between July 9 and August 11 (Fig. 3). The mean date was July 20, and in the majority of years first setting took place between July 16 and 22. This finding supports broadly the formula which was offered earlier by Loosanoff and Nomejko (1951) who stated (p. 124) that "The beginning of oyster setting in Long Island Sound should be usually expected on July 19 ±4 days, regardless of lunar phases and of changes in hydrostatic pressure caused by changes in the tidal level." The exceptions to the rule were the years of 1949, 1951, and 1952, when setting began between July 9 and 12, and 1959, the only year when the beginning of setting was recorded later than the end of July (Fig. 3).

The observations on setting of oysters were made in some years from the intertidal zone to the depth of 100 feet (Table I). Setting was recorded at all depths but, as with starfish (Loosanoff, 1964), the duration of the setting at the deepest stations was shorter than at the shallower ones.

As a rule, setting began at all the stations in 30 feet of water or less within the

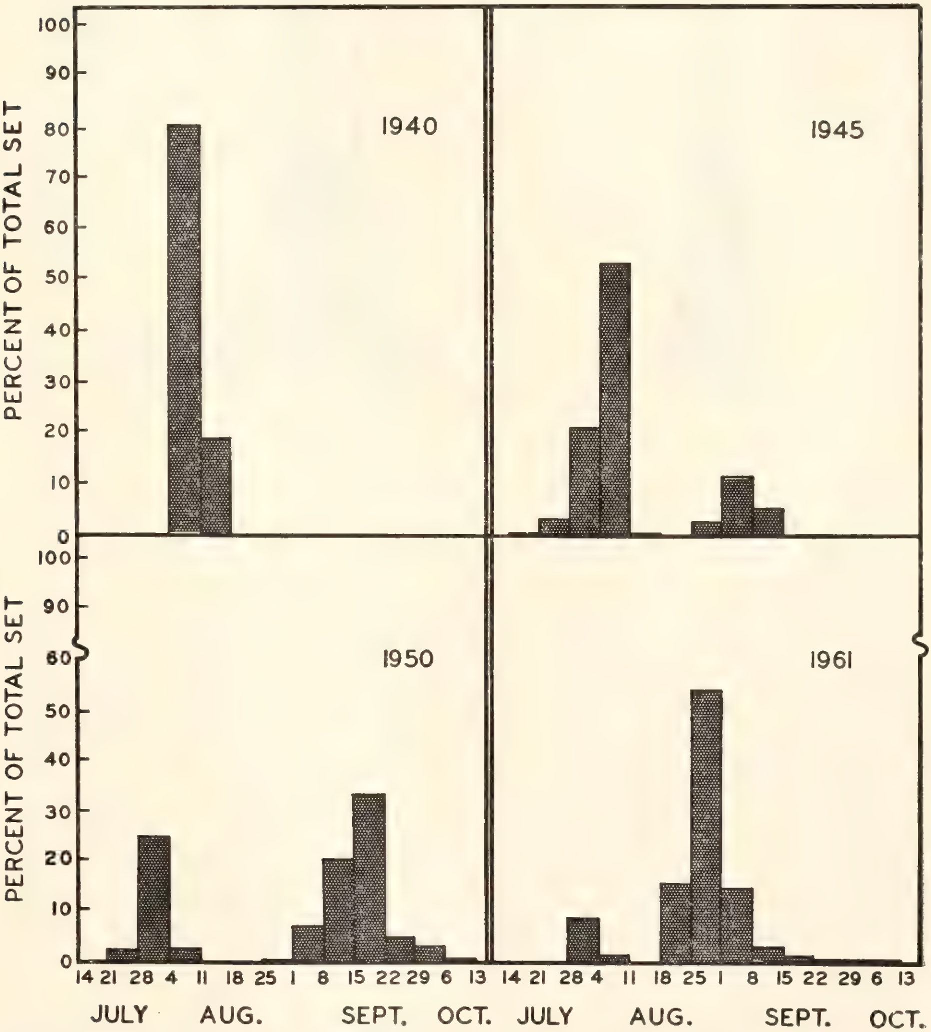

FIGURE 4. Variations in weekly intensity of oyster setting in different years. In 1940 99.76% of the entire set occurred within two weeks of the first wave, but in other years setting was more evenly distributed through the summer and two definite waves were distinguishable.

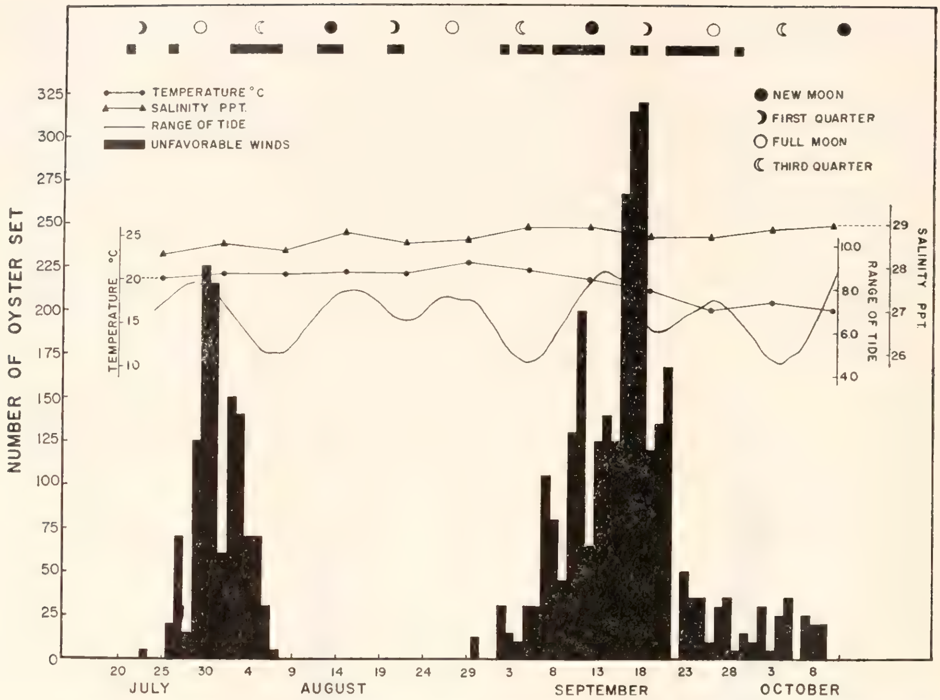

FIGURE 5. Average daily number of oyster set per station recorded in 100 oyster shell-surfaces in Long Island Sound, 1950. Two definite waves of setting, separated by a "dead period," can be seen. Some of the environmental conditions are also shown.

same 24-hour period. Even at stations located as deep as 50 or 60 feet, setting started at approximately the same time as at shallower stations.

As found in a parallel study on propagation of another invertebrate of Long Island Sound, the starfish (Loosanoff, 1964), the length of the setting period of oysters varied greatly in different years (Fig. 3). The shortest time between the beginning and end of setting was in 1957, only 36 days; while the setting period of the preceding year, 1956, extended for 85 days. In nine years the duration of setting was 70 days or longer, and the average length of the setting period was 65 days, or about two months.

The earliest date for the end of setting was August 26, 1957. This year, as mentioned earlier, had the shortest setting period. The latest date was October 17, 1956, when the period of setting was the longest within the period of our studies. The mean date for the end of setting was September 22. The difference between the earliest and the latest dates for the end of setting was 51 days, considerably larger than the difference of 34 days for the earliest and latest dates of the beginning of setting.

Although the average setting period of Long Island Sound oysters was about 65 days, the average length of the setting period of starfish during the same period was about 52 days. Thus, the lengths of the setting periods of these two invertebrates living under identical conditions do not differ radically.

The length of the setting period of oysters given here merely shows the number of days between the beginning and the end of setting. It does not mean that setting was continuous or of uniform intensity during this entire period. In reality, setting ceased for various (sometimes lengthy) periods almost every year. Such a "dead period," for example, occurred in 1950 when not a single oyster larva set on any of our collectors between August 7 and 30 (Fig. 5). This gap appeared even though the number of mature oysters in Long Island Sound then amounted to many millions. During most of this period the water of the Sound was colored somewhat pinkish by plankton blooms. A dinoflagellate, *Prorocentrum triangulatum,* was one of the common organisms present. In 1957 setting was recorded only on the first day and then 36 days later on the last day, while between these dates no setting occurred. The years 1958 and 1959 were exceptional because setting was continuous during practically the entire season (Fig. 3).

Further differences and peculiarities of setting in different years are illustrated in Figure 4. For example, the setting season extended for 70 days in 1940, but over 99% of the set was between August 5 and 18; the additional fraction of about 1% was spread so thinly through the remainder of the setting season that it cannot even be clearly shown in the illustration. Thus, during that year almost the

TABLE II

Average numbers of total oyster set per station per 100 shell-surfaces, and numbers of set recorded during each of the two waves, Long Island Sound, 1937–61

Year	Total set	Total 1st wave	Total 2d wave
1937	1640	1621	19
1938	1090	83	1007
1939	42623	42230	393
1940	42407	42378	29
1941	17225	15192	2033
1942	2138	71	2067
1943	72	57	15
1944	11147	10407	740
1945	4476	3530	946
1946	3267	1851	1416
1947	1136	438	698
1948	344	151	193
1949	739	730	9
1950	405	121	284
1951	1181	170	1011
1952	998	684	314
1953	2338	102	2236
1954	77	24	53
1955	10915	386	10529
1956	2289	1884	405
1957*	3	0	0
1958	15417	13903	1514
1959	2312	2250	62
1960	780	702	78
1961	234	23	211

* Only stations 1, 2, and 3, all in the Milford area, were maintained in 1957.

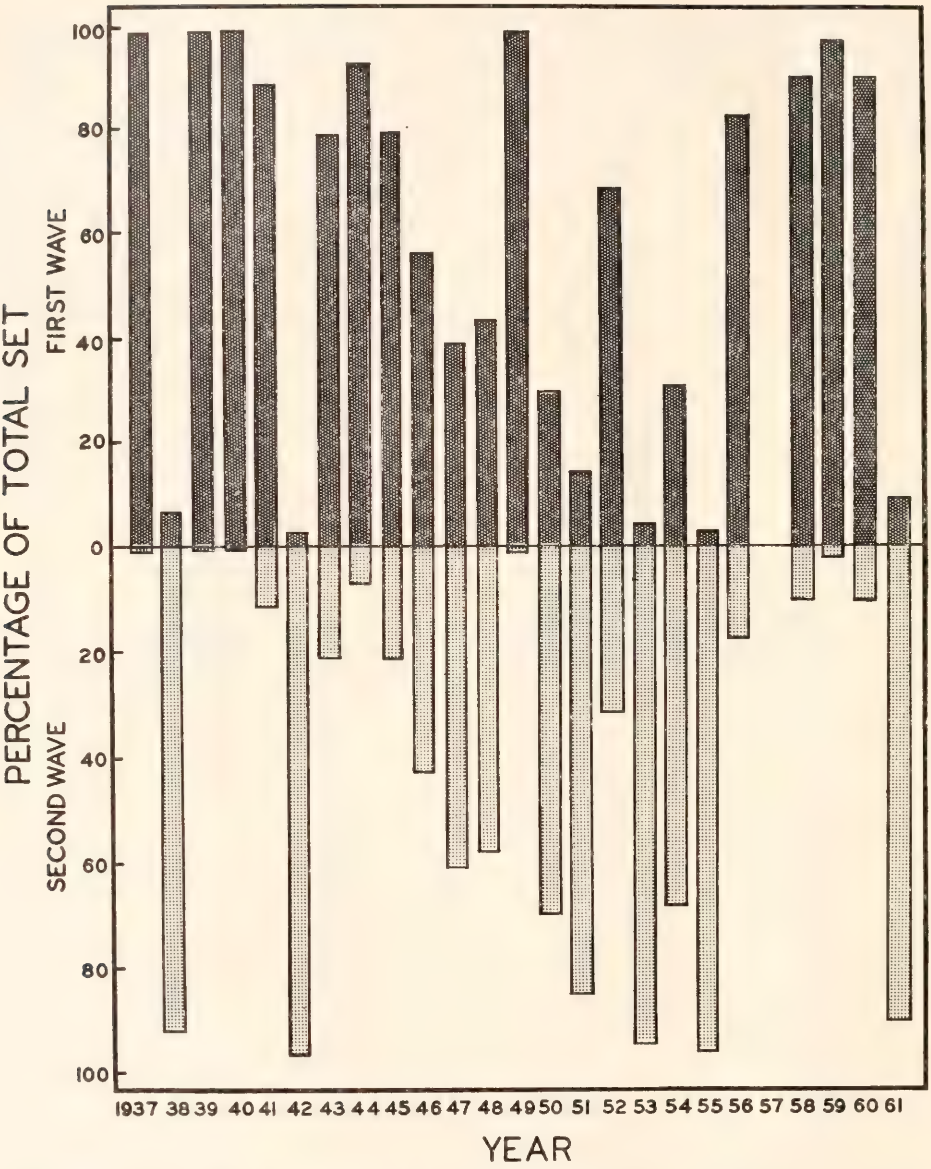

FIGURE 6. Percentage of total oyster set in each of the two waves during different summers, Long Island Sound, 1937–61.

entire setting occurred in a single wave of short duration. In 1945, however, setting took place in two waves, of which the first was the heavier. In contrast, during 1950 and 1961 the first wave of setting was the lighter of the two and the intensity of setting was much more evenly distributed throughout the setting period than in 1940 (Fig. 4).

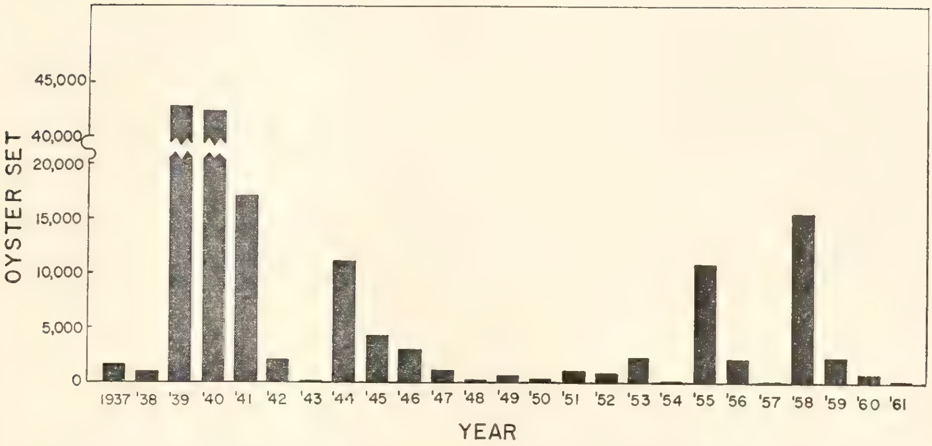

FIGURE 7. Average number of oyster set per station recorded in different years on 100 oyster shell-surfaces, Long Island Sound, 1937–61.

TABLE III

Average number of oyster set per station in different years on 100 oyster shell-surfaces, at stations located at 10-, 20-, and 30-foot depths, Long Island Sound, 1937–61

Year	Depth in feet		
	10	20	30
1937	2050	2175	695
1938	245	935	2090
1939	23945	45925	58000
1940	24635	54845	28200
1941	11454	21212	18515
1942	1000	2093	3345
1943	65	81	35
1944	24700	1775	2447
1945	6348	4649	1774
1946	1748	6508	2053
1947	1109	1467	842
1948	314	542	184
1949	783	585	833
1950	278	540	440
1951	848	707	2098
1952	1395	1283	183
1953	615	3028	3945
1954	74	95	63
1955	3660	7031	24473
1956	1641	1983	3457
1957*	5	5	0
1958	12977	32581	1505
1959	2216	3723	1030
1960	612	1376	406
1961	391	240	20

* Only stations 1, 2, and 3, all in the Milford area, were maintained in 1957.

Again we see a similarity in some aspects of setting of oysters and starfish of Long Island Sound because the latter also show considerable variation in intensity of setting during different summers. Sometimes the entire set may occur within a single week or 10 days, whereas in other years setting continues for a long time although the intensity may vary greatly from week to week (Loosanoff, 1964).

The beginning of the first wave of oyster setting varied from July 9 (1952) to August 11 (1959), and had a mean of approximately July 20 (Fig. 3). The earliest end of the first wave was July 21 (1957), a year of extremely poor setting, and the latest was September 17 (1959); the mean was approximately August 12. The date of the beginning of the second wave varied from August 7 (1951) to September 18 (1959), and the average was August 23; the end of the second wave which, of course, was the end of the setting season, came between August 26 (1957) and October 17 (1956); the average was about September 23.

Table IV

Rank-order of years in relation to the intensity of setting of oysters at stations of three different depths, Long Island Sound, 1937–61

Year	Rank-order of years at each depth (feet)				Year	Rank-order of years at each depth (feet)			
	10	20	30	All stations		10	20	30	All stations
1937	9	10	17	13	1950	21	21	18	20
1938	22	17	10	16	1951	15	18	9	14
1939	3	2	1	1	1952	12	16	21	17
1940	2	1	2	2	1953	17	9	5	9
1941	5	4	4	3	1954	23	23	22	23
1942	14	11	7	12	1955	7	5	3	6
1943	24	24	24	24	1956	11	12	6	11
1944	1	13	8	5	1957*	25	25	25	25
1945	6	7	12	7	1958	4	3	13	4
1946	10	6	11	8	1959	8	8	14	10
1947	13	14	15	15	1960	18	15	19	18
1948	20	20	20	21	1961	19	22	23	22
1949	16	19	16	19					

* Only stations 1, 2, and 3, all in the Milford area, were maintained in 1957.

The peaks, or maxima, of the waves also varied considerably from year to year. Their approximate dates are indicated in Figure 3 by cross-bars. The peaks of the first wave came between July 14 (1952) and August 29 (1960); the approximate average was July 30. The second wave had peak dates from August 19 (1952) to September 23 (1959), and an average of September 4. The difference in days between the maxima of the two waves ranged from 19 days (1960) to 40 days (1943).

Neither the first nor the second wave of setting was consistently the predominant one. Table II, giving average numbers of total oyster set per station for the two waves in each year, shows that each wave was dominant in a number of years. In some years almost 100% of the annual set took place during the first wave, but in others most setting was later in the season, during the second wave (Fig. 6).

Within the 25-year period the first wave was the heavier in 15 years and the second in 10.

In most years the heavier of the two waves predominated decidedly. Nevertheless, there were few years when the percentage of total set during the season was more or less evenly divided between the two waves. The years 1946, 1947, and 1948, when about a 40:60 ratio existed, were closest to such even distribution (Fig. 6).

The intensity of setting differed considerably from year to year. It was lowest in 1957, when only about three oysters per 100 shells were counted at each station during the entire season; in 1939 and 1940 over 42,000 spat were counted under identical conditions (Fig. 7, Table II). A comparison of the numbers of oyster set at the stations of the three different depths showed that no one depth consistently produced the heaviest set (Table III).

Each group of stations, whether at the 10-, 20-, or 30-foot depth, occupied the first, second, or third rank during one or more years of our observations. If all stations, regardless of depth, were considered, then, as already mentioned, 1939 was the year of heaviest setting, and 1957 was the poorest. Stations of different depths differed in this respect, however. For example, in 1939 the stations at the 30-foot depth had the heaviest set of the entire 25-year period (Table IV). For the 10-foot stations, however, 1939 ranked third, and for the 20-foot stations it ranked second.

In some other years the differences in intensity of setting at stations of different depths varied even more. Thus, 1944 was the best year for the stations at 10-foot depth, but for 20-foot stations it ranked 13th and for the 30-foot stations, 8th. For all the stations taken together regardless of their depth it ranked the 5th heaviest year in 25. In some years, nevertheless, as in 1943, the annual rank-order was the same at three different depths (Table IV).

I wish to thank my associates at the Milford Biological Laboratory who helped in collection of the data on which this article is based—especially my former biological aides, Charles Nomejko, John Malarkey, and James B. Engle, who examined the spat collectors. Joseph F. Lucash and Herman R. Glas captained the research boats. Mrs. Susan Webb prepared the illustrations and otherwise helped in completion of the manuscript. Harry C. Davis examined plankton samples collected during the last few years of these studies. Dr. Ralph Hile, Mr. Davis, and Miss Rita Riccio made helpful editorial suggestions.

SUMMARY

1. Studies on setting of oysters in Long Island Sound during a 25-year period, 1937–61, have shown that the larvae of advanced stages were never extremely common and were unevenly distributed in the water masses. Their appearance and disappearance did not follow a definite pattern.

2. No clear relation was found between the number of certain larval enemies, such as ctenophores, and intensity of setting. Neither could the numbers of larvae be correlated with the numbers of adult oysters.

3. Dead or dying larvae were sometimes found in plankton. Disappearance of the larvae possibly may be due to the plankton blooms that produce detrimental external metabolites. A dinoflagellate, *Prorocentrum triangulatum,* on occasion present in extremely large numbers, may be one of the forms responsible for the absence of larvae during certain periods.

4. Setting of oyster larvae was recorded at all depths from the intertidal zone to 100 feet.

5. Beginning of setting at all stations at the depth of 30 feet or less normally occurred within the same 24-hour period.

6. The average length of setting season of oysters in Long Island Sound was 65 days. The shortest season was 36 days, and the longest was 85 days.

7. Setting in different years started between July 9 and August 11; the mean date was about July 20. The earliest end of setting was August 26, and the latest, October 17.

8. The setting in Long Island Sound is not continuous or of uniform intensity. Setting ceased almost every year for various periods, sometimes several weeks long.

9. Setting exhibited two well-defined waves in most years; each peak had a clearly evident maximum. The time these peaks appeared varied considerably from year to year. The time between the peaks of two waves in different years ranged from 19 to 40 days.

10. Neither the first nor second wave of setting was consistently predominant. Within the 25-year period the first wave was the heavier in 15 years, and the second in 10.

11. The intensity of setting in the same areas of Long Island Sound differed widely from year to year. Setting was lowest in 1957, when approximately three recently set oysters per 100 shell-surfaces were counted at each station during the entire season; in 1939 and 1940 over 42,000 spat were counted under the same conditions.

12. Comparison of the numbers of oyster set at stations of three depths—10-, 20-, and 30-feet—showed that no depth consistently produced the heaviest set. Stations of each of the three depths had the greatest abundance of larvae during one or more years of the period of observations.

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AET AND RADIATION-INDUCED CROSSING-OVER IN MALE DROSOPHILA MELANOGASTER[1]

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AET, S, 2-aminoethylisothiouronium Br, has reported to be one of the most efficient sulfhydryl compounds that will protect mice against death from radiation (Doherty and Burnett, 1955). The various hypotheses suggested for the mechanism of protection include those that should protect chromosomes from radiation-induced damage. Induced crossing-over in male *Drosophila,* or more properly "pseudo-crossing-over" to indicate induced breakage, was one of the radiation-induced chromosomal aberrations selected to test whether AET would protect against this effect. Lefevre (1947) with work on radiation-induced somatic crossing-over, and Parker (1948) and Whittinghill (1951) from evidence with induced crossing-over in the male *Drosophila* interpreted this as a result of chromosomal breakage. Herskowitz and Abrahamson (1957), Olivieri and Olivieri (1964) related dosage to amount of induced crossovers and concluded that a "two-hit" phenomenon was responsible.

MATERIALS AND METHODS

Adult male *Drosophila melanogaster* 2–16 hours old, heterozygous for third chromosome roughoid (*ru,* 0.0), hairy (*h,* 26.5), thread (*th,* 43.2), scarlet (*st,* 44.0), curled (*cu,* 50.0), stripe (*sr,* 62.0), ebony (e^s, 70.7), claret (*ca,* 100.7), as a result of cross between Oregon-R and "rucuca," were injected with 3 mg. of AET/ml. in 0.01 *M* NaOH. The AET was converted to effective MEG with a pH of 7.2. The injection was done with a glass needle with a tip diameter of 0.06 mm. The amount of fluid introduced between the dorsal third and fourth tergites was determined by increase in the weight of the flies and was found to be approximately 0.1 μl. or 3.33×10^{-5} mg. of AET per fly. The *Drosophila* males, usually in two groups of 25, one a control group injected with saline and the other treated with AET, were exposed immediately after injections to 2000 r of x-ray radiation in air by a Mattern x-ray unit with a Thermax tube at 100 KV, 5 ma., $\frac{1}{2}$ mm. of Al filter, 320 r/min. (determined by a Victoreen Model 570 condenser r-meter) at a distance of 11 cm.

In one series, 120 pretreated AET males and 101 control males were irradiated and allowed to recover in air and then mass-mated to virgin "rucuca" at a ratio of one male to three females, aged two to three days. The males were presented with a virgin group of females every three days. On the ninth day after irradiation, each male was isolated in a small vial and given three females, and on the 12th day this was repeated in a new vial of food. Thus, the offspring of each male could be recorded for 9–12- and 12–15-day broods after irradiation.

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In another series 212 males were injected with AET and a control group of 125 were exposed to 2000 r in air. Then they were both post-treated with flowing N_2 for 30 minutes. The flies were mated at a ratio of one male to three females in broods 0–3 day, 3–6 day and then mated daily on days 6 through 15. All experiments were kept at 24° C. The design of the experiments was based on the consensus of several authors (Auerbach, 1954; Bateman, 1956; Savhagen, 1961) that the cells in meiosis at the time of radiation of young adult males' testes will be mature usable spermatozoa 7–9 days later. Thus, induced crossing-over in diploid cells should appear in the offspring resulting from matings 7 or 8 days after irradiation. Crossovers as suggested by Auerbach (1954) were scored when at least two or more mutants (r_2) were present, for presence or absence of a single mutant (r_1) could represent a deletion or mutation.

TABLE I

Radiation-induced crossovers in adult males 2–16 hours old, heterozygous for ru h th st cu sr e^s ca, injected with 3 mg./ml. of AET, and then irradiated with 2000 r of x-rays and recovered in air.

Brood	AET				Control			
	Crossover		Total	r_2% Total gametes	Crossover		Total	r_2% Total gametes
	r_1	r_2			r_1	r_2		
9–12-day	7	50	8509	0.588%*	8	45	5078	0.886%
12–15-day	13	41	11415	0.359%	10	19	4187	0.453%

* $\chi^2 = 4.02$.

RESULTS

The males treated with AET and then irradiated had a significant decrease in the number of r_2-type crossovers in the 9- to 12-day brood as compared to offspring from the control males (Table I). By means of a 2 × 2 contingency table comparing crossovers to total offspring the chi square was calculated to be 4.02. However, there was no significant difference between the 12–15-day brood of the pretreated AET males and control males. Another method of presenting the data, namely the percentage of males induced to produce crossovers, was considered. Although this method would avoid to some extent the problem of clusters, the difficulty would be in giving equal status to males with considerable differences in the number of their offspring. Thirty-nine out of 118 AET-treated (33.1%) males produced crossovers in the 9–12-day brood, while 25 out of a total 87 fertile control males (28.7%) had crossovers in their offspring. In the 12–15-day brood there were 17.4% and 16.6% males, respectively, induced to produce crossovers. Although the 12–15-day brood represents spermatogonia that may have undergone further divisions, and thus produced clusters, the number of crossovers per thousand flies (gametes) was less in this brood in both the AET and control offspring than in 9–12-day brood. Non-crossovers are also produced in clusters.

The analysis of the induced crossovers as to the region in which breakages occurred (if it is indeed the case) revealed that the majority of them were in region

TABLE II

Location and number of breaks in radiation-induced (2000 r) crossovers (r_2) in males whose third chromosome was heterozygous for ru h th st cu sr e^s ca. Radiation in air and recovery in air. Location of regions of breakage: ru I *h* II *th* III *st* IV *cu* V *sr* VI *e^s* VII *ca*

Region	Number of breaks			
	AET (3 mg./ml.)		Control	
	9–12-day	12–15-day	9–12-day	12–15-day
I	1			
II				
III			1	
IV	43	40	41	19
V	5	1	2	
VI	1	1	3	
VII	1		5	
Non-crossover	8452	11361	5025	4158

IV between *st* and *cu* and included the centromere (Table II). AET did not influence the region of the breakage, for in the control 9–12-day brood, 80.3% of the breaks occur in this centromere region, while in the offspring of AET-treated males, 84.3% of all breaks occur in this same region. The 12–15-day broods had a largest percentage of breaks about the centromere.

The series on nitrogen post-treated was conducted to test the hypothesis that the AET affected the recovery system. The data in Table III do indicate that post-treatments in nitrogen tend to reduce the number of crossovers produced by

TABLE III

Radiation-induced crossovers in adult males 2–16 hours old heterozygous for ru h th st cu sr e^s ca' injected with 3 mg./ml. of AET irradiated with 2000 r in air and post-treated with N_2 for 30 minutes

Brood	AET				Brood	Control			
	Crossover		Total	r_2% Total gametes		Crossover		Total	r_2% Total gametes
	r_1	r_2				r_1	r_2		
day 6	1	0	1010		day 6	1	0	1328	
day 7	1	3	672	0.446	day 7	1	1	841	0.119
day 8	0	2	736	0.272	day 8	1	5	1274	0.392
day 9	1	17	2643	0.681	day 9	0	19	2582	0.736
day 10	2	14	2669	0.525	day 10	3	13	1459	0.891
day 11	1	14	3010	0.465	day 11	3	13	2182	0.596
day 12	4	21	3376	0.622	day 12	0	2	2560	0.078
day 13	1	3	3358	0.089	day 13	8	6	2432	0.247
day 14	0	18	2303	0.782	day 14	0	9	1403	0.641
day 15	0	1	1246	0.080	day 15	0	4	1249	0.320

the control males so that there is no significant difference between 9–12-day brood of control males and that of AET and post-treated nitrogen males with respect to the number of crossovers. Although the data were presented of the daily broods, the three daily broods of days 9, 10, and 11 were totaled and then compared to the 9–12-day broods of flies recovered in air. The daily broods of days 12, 13, and 14 were also totaled and compared to 12–15-day broods of flies recovered in air. By this method the daily brood data presented in Table III were analyzed and the 9–12-day brood of control males treated in N_2 produced 45 (0.723%) crossovers out of a total of 6225. This is lower than 0.886% crossovers in the corresponding brood recovered in air (Table I). The 9–12-day brood of AET and post-treated N_2 males yielded 45 (0.541%) crossovers in a total of 8322 which is quite similar to 50 (0.588%) crossovers in a total of 8509 in the 9–12-day brood of AET and air-recovered males. The control 12–15-day brood as a result of post-treatment with N_2 had 17 crossovers in 6395 (0.266%) while the same brood treated with AET and post-treated with nitrogen, 42/9037 (0.456%) which is chi square difference of 3.85. The nitrogen post-treatment reduced the amount of crossovers in control 12–15-day brood but not the percentage of crossovers in the broods of males treated with AET. The region of the breakage was not affected by the post-treatment of N_2 (Table IV) and the breakage was most frequent about the centromere.

TABLE IV

Location and number of breaks in radiation-induced (2000 r) crossovers (r_2) in males whose third chromosome was heterozygous for ru h th st cu sr e^s ca. Radiation in air, post-treated with nitrogen for 30 minutes. Location of regions of breakage: ru I *h* II *th* III *st* IV *cu* V *sr* VI *e^s* VII *ca*

Brood-day	AET (3 mg./ml.)		Control	
	Number of breaks, region	Total gametes	Number of breaks, region	Total gametes
6	0	1009	0	1327
7	1 I, 1 IV, 2 V, 1 VIII	668	1 I, 1 III, 1 V, 1 VII	839
8	2 IV	734	5 IV	1268
9	17 IV	2625	18 IV, 1 V	2563
10	13 IV, 1 VI,	2653	12 IV, 1 V	1443
11	1 I, 1 III, 12 IV, 1 V, 1 VII	2995	1 III, 13 IV, 1 VI	2169
12	21 IV	3351	2 IV	2558
13	3 IV	3354	6 IV	2418
14	9 IV	2285	18 IV	2285
15	4 IV	1123	1 IV	1245

DISCUSSION

The x-ray-induced crossing-over in young adult male *Drosophila* resulted in a greater percentage of crossovers in the older spermatogonia (those nearer to meiosis) than in the younger spermatogonia. The older spermatogonia represented by 9–12-day brood indeed may be a more sensitive period for induced crossing-over.

It is also possible that young spermatogonia were more susceptible to injury and hence failed to survive, and thus there were fewer crossovers. There is also a relatively smaller population of spermatogonia represented by 12–15-day brood at the time of radiation. Olivieri and Olivieri (1964) also found that radiation induced more crossovers in the late spermatogonia than the early spermatogonia.

The question raised by the data in Table I is whether AET actually protects the 9–12-day brood from crossing-over, or whether the broken or damaged chromosomes are prevented from repairing or rejoining, hence are lost. From the evidence in the literature AET and other sulfhydryls apparently do not give protection to radiation-induced chromosomal aberrations in *Drosphila*. Edington (1958) reported that when AET was used as a possible protector against radiation-induced mutations in male *Drosophila,* there was an increase in "dominant lethals" and in the numbers of sex-linked lethals (Kaplan and Lyon, 1953). Mittler (1964) found that pretreatment with MEA, AET, and glutathione did not protect the *Drosophila* testes from radiation-induced sex-linked lethals, translocations, deletions of the X chromosome, loss of X or Y chromosomes and "dominant lethals," and that glutathione and MEA increased the number of "dominant lethals" and loss of chromosomes in the XO method. Rugh and Fu (1965) reported that AET did not protect the cytoplasm or nucleus of the haploid *Arbacia* cell from gamma rays.

The region that includes the centromere was found to have the majority of the breakages or exchanges that occurred. Olivieri and Olivieri (1964), Friesen (1937), Parker (1948) and Whittinghill (1951) also reported that induced crossing-over is greatest in this centromeric heterochromatic region. AET or post-treatment with N_2 did not influence the breakage in this region.

The paradox of a radioprotective substance actually increasing radiation damage may be explained by AET having a greater adverse effect on the recovery process than on actual protection, if any, against the initial effect. It may be that 2000 r may so overwhelm the initial protective effect of AET, if any, that only the effect on the recovery system is tested. For this reason, the series on nitrogen post-treatment was conducted. If one assumes that AET induces anoxia or by some means prevents the recovery of broken chromosomes, then control males whose testes were injured by radiation in air and who were then subjected to nitrogen should yield data similar to AET-treated males recovered in air and thus have less crossovers.

The data in Table III do indicate that post-treatment with nitrogen tended to reduce the number of crossovers produced by the control males so that there is no significant difference between 9–12-day brood of control males and that of AET-treated males with respect to the number of crossovers. Since post-treatment with N_2 does induce anoxia and since the nitrogen post-treatment has no effect on AET-treated males with respect to a change in the number of crossovers, it appears as if AET injection induces an anoxia condition which simulates to some extent the post-nitrogen treatment.

Oliveri and Oliveri (1964) were surprised to find an increase in crossing-over in males irradiated in air and post-treated in N_2, for they had also noted that N_2 when given between the fractions restored the frequency of the induced crossing-over as if it were unfractionated, and that N_2 acted by preventing the restitution of breaks. The data presented in this paper indicated that N_2 post-treatment tends to reduce the number of crossovers and that the day 10 brood used only by the Olivieris to test the effect of post-treatment of N_2 produced the largest percentage of crossovers in our control group post-treated with nitrogen (Table III). The young spermatogonia, broods day 12–15, were definitely affected by the post-treatment of N_2. The 30 minutes of N_2 treatment were sufficient to decrease the number of crossovers, probably by keeping the broken chromatids or chromosomes from uniting with a sister pair, hence less crossovers. If the broken or injured chromosomes are lost as a result of the N_2 treatment, this would also result in a decrease in radiation-induced crossovers. However, AET does not affect the younger spermatogonia, for the decrease in crossovers was in the older spermatogonia. It is indeed possible that something besides anoxia induced by AET may be interfering with the recovery process. Sobels (1963) pointed out the extreme difference in response of the mature spermatozoa compared to that of spermatids with respect to N_2 post-treatments. Not only are there differences in radiosensitivity for the various pre- and post-treatments. Even a difference of several hours in the age of mature sperm can result in significant differences in radiation-induced lethals (Lefevre and Jonsonn, 1964).

AET does not protect against breakage of chromosomes or whatever radiation-induced damage causes crossing-over in male *Drosophila*. Although there are fewer induced crossovers in the 9–12-day brood which represents cells about to go into meiosis, the AET effect is eliminated by post-treatment with N_2. The induced crossovers of N_2 post-treated males, whether injected with AET or saline, will be lower as a result of the N_2 treatment.

The excellent technical assistance of Raymond U is acknowledged.

SUMMARY

1. *Drosophila melanogaster* males heterozygous for *ru h th st cu sr* e^s *ca* were injected with AET and irradiated with 2000 r and backcrossed to "rucuca." The number of induced crossovers with two or more mutants (r_2) was significantly decreased in the 9–12-day broods of males treated with AET as compared to controls.

2. The hypothesis that AET interfered with a recovery process probably by an induced anoxia was tested by N_2-treatment after the x-ray-treatments. The N_2 post-treatments had no effect on AET-treated and irradiated males with respect to crossovers; however, the young spermatogonia represented by brood day 12–15 of the control males were induced by the N_2 treatment to produce less crossovers.

3. Since the AET effect is eliminated by N_2 post-treatment, an anoxia production by AET can be a possible mechanism for reduction of crossovers. Nitrogen post-treatment tends to reduce the number of radiation-induced crossovers. AET did not affect the young spermatogonia.

4. The induced breakage occurred primarily between regions *st* and *cu* and thus in or about the centromeric region. AET has no influence on the region of the breakage.

5. Crossovers were found to be more frequent in the 9–12-day brood than in the 12–15-day brood.

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THE JUVENILE HORMONE. V. THE SENSITIVITY OF THE BUG, PYRRHOCORIS APTERUS, TO A HORMONALLY ACTIVE FACTOR IN AMERICAN PAPER–PULP[1]

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When reared in the Biological Laboratories at Harvard University, the European bug, *Pyrrhocoris apterus* L., failed to undergo normal metamorphosis. In the initial cultures, 1,215 individuals showed normal growth and moulting until the fifth larval instar. Then, instead of metamorphosing into adults, all except one individual underwent a supernumerary larval moult to form "perfect" sixth instar larvae or adultoid sixth instar larvae (Table I). All the perfect sixth instar larvae underwent yet another supernumerary moult to form even larger seventh instar larvae or seventh instar adultoid larvae. Of the entire group of 1,215 larvae, only one individual completed metamorphosis and attained sexual maturity.

Among tens of thousands of *Pyrrhocoris* which one of us (K. S.) had cultured in Prague, the spontaneous formation of a sixth instar had never been encountered. Metamorphosis had always taken place at the end of the fifth larval stage—a result directly attributable to the inactivation of the corpora allata and the cessation of juvenile hormone secretion at the outset of the fifth instar (Sláma, 1962). The formation of sixth stage larvae had been provoked only in experiments in which juvenile hormone was supplied by the implantation of active corpora allata (Novák and Sláma, 1961; Sláma, 1964a). For these several reasons, it seemed certain that the Harvard cultures of *Pyrrhocoris* had access to some unknown source of juvenile hormone.

One possibility was that some feature of the Harvard environment served to prevent the normal inactivation of the animal's own corpora allata and that, in this indirect manner, metamorphosis was blocked by the prolonged and abnormal secretion of juvenile hormone. An alternative possibility was that the Harvard cultures were under the influence of an environmental factor which, in itself, possessed juvenile hormone activity.

The present study initiates a detailed investigation of the phenomenon.

MATERIALS AND METHODS

1. *Experimental animals*

Pyrrhocoris apterus (Heteroptera; Pyrrhocoridae) is a typical hemipteran found throughout Europe feeding by its sucking mouth-parts on the seeds of lime

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(linden) trees (*Tilia* sp.). The insect is not known to occur in the United States or Canada.

A subculture of *Pyrrhocoris apterus* was obtained from the Prague Laboratory of Insect Physiology and brought to Harvard by permission of the United States Department of Agriculture. The experimental cultures were reared in 10-cm Petri dishes in a 25° C incubator programmed for a daily illumination of 17 hours. Each Petri dish contained a supply of linden seed (*Tilia cordata*), along with a cotton-plugged vial of tap water. To increase the surface available to the bugs, each dish also contained a folded strip of paper towelling (Scott Paper Co., Brand 150).

Certain experiments were performed on two other species of Heteroptera—*Rhodnius prolixus* and *Oncopeltus fasciatus*. *Rhodnius* was obtained from Dr. Andrew Spielman, of the Harvard Medical School, and Mr. Paul Toselli, of the Yale Medical School; *Oncopeltus* was supplied by Prof. A. Glenn Richards, of the University of Minnesota.

TABLE I

Supernumerary larval instars in the Harvard cultures of Pyrrhocoris apterus

5th instar larvae moulted into:	Number of animals
Perfect or almost perfect 6th instar larvae*	500
Adultoid 6th instar larvae	714
Normal adults	1

* Many individuals underwent a further moult to form 7th instar larvae or adultoids.

Certain other experiments were performed on five species of silkworm pupae, namely, *Hyalophora cecropia*, *H. gloveri*, *Antheraea polyphemus*, *A. mylitta*, and *Samia cynthia*. The first three species were stored at 5° C; the last two at 8° C. In order to induce adult development, the previously chilled pupae were placed at 25° C.

2. *Allatectomy*

Corpora allata were removed from *Pyrrhocoris* by the technique described by Sláma (1964b).

RESULTS

1. *Anatomical and behavioral studies*

Of the large number of abnormal *Pyrrhocoris* reared at Harvard, a total of 435 were subjected to detailed examination and dissection. The results were as follows:

Those sixth instar larvae which showed no trace of adult characters underwent a second supernumerary moult after five further days. The seventh instar larvae were often unable to escape from the exuviae and, in that event, they soon died. The females, on dissection, showed juvenile ovaries without any ripened eggs; by contrast, the seminal vesicles of the males were full of mature spermatozoa.

Those sixth instar larvae which showed only a few patches of adult cuticle also tried to moult again. However, the old cuticle remained attached to the epidermis at the sites of the patches of adult cuticle. Consequently, all these individuals died without completing the ecdysis.

The adultoid sixth instar larvae behaved in one of two ways. In the process of moulting to the sixth instar, the majority soon died without being able to detach the exuviae from the appendages or other parts of the body. A lesser number completed the ecdysis and then survived for one to three months without any further moulting. On dissection, the thoracic glands were found partly or wholly degenerate. Here, again, the males showed mature spermatozoa, while, surprisingly enough, the females contained ripe, chorionated eggs which, in the absence of functional genitalia, could not be oviposited. Since feeding is impossible when the abdomen is distended with ripe eggs (Sláma, 1964c), all these females died within a month or so.

The adultoid sixth instar larvae showed bizarre behavior. Despite the absence of external genitalia, the adultoid females were extremely attractive to the adultoid males and the latter made repeated efforts to copulate. So also, the adultoid males pursued and tried to mate with normal females, while normal males tried to mate with adultoid females. Needless to say, all these efforts were unsuccessful in the absence of genitalia. These findings point to the secretion of sex pheromone by the adultoid females and the maturation of adult sexual behavior by the adultoid males.

2. *The environmental factor*

A detailed comparison of the culture conditions at Harvard *versus* Prague suggested 15 differences. Attention first centered on the fact that the Harvard cultures were stored in incubators which also contained silkworm pupae at various stages of adult development. We were mindful of Loher's (1960) finding that the activity of the corpora allata of the desert locust can be sustained by a volatile emanation from other locusts. It seemed altogether likely that we had encountered an analogous interaction between *Pyrrhocoris* and the developing silkworms. Appropriate experiments were performed to test this hypothesis. All were negative.

We also examined the possibility that the Petri dishes, or the seed, or the water-vials, or the water itself had somehow become contaminated with one or more of the potent preparations of juvenile hormone under simultaneous study in the Harvard laboratory. No evidence could be found to support any of these propositions.

By a process of elimination, attention finally focused on the fragment of paper towelling which had been placed in each Petri dish. In Prague, filter paper had routinely been used in the cultures. We were astonished to find that when the towelling was replaced by a corresponding fragment of Whatman's filter paper, the entire phenomenon vanished and all individuals developed normally.

3a. *Scott, Brand 150*

The just-mentioned finding seemed incomprehensible. In correspondence with the Philadelphia laboratory of the Scott Paper Company, we sought to inform ourselves as to the chemicals added to Brand 150. All we learned was that the towelling was made from paper-pulp.

3b. *Tests of other paper products*

Twenty other brands of towelling, napkins, and bathroom tissues were assembled and tested by allowing freshly moulted, fifth instar *Pyrrhocoris* larvae to walk upon them. The following brands showed high juvenile hormone activity as signaled by the moulting of all individuals into sixth instar larvae or adultoid forms: *Scottowels, Scott Tissue Service Roll, Scott Cut-Rite Wax Paper, Scotties, Scotkins Paper Napkins, Kleenex, Kleenex Two-Ply Tissues, Kleenex Bathroom Tissues, Kleenex Dinner Napkins, Hudson Table Napkins, Kimwipes, Doeskin Facial Tissues.*

These extraordinary findings suggested that the active factor was widespread in paper products. Therefore, additional tests were performed on newspapers and journals. In each case, a fragment of the material was placed in the culture dishes in contact with freshly moulted, fifth instar *Pyrrhocoris* larvae.

The *New York Times, Wall Street Journal, Boston Globe, Science,* and *Scientific American* proved to be even more active than the above-mentioned towelling. By contrast, the *London Times* and *Nature* were completely inert, and so were a number of other paper materials of European or Japanese manufacture. Paradoxically, Japanese newspapers were as active as American ones. However, we later learned that Japanese newsprint is imported from Canada.

The conclusion was self-evident: American paper products contain an active material which blocks the metamorphosis of *Pyrrhocoris*. European or Japanese paper products are apparently deficient in this factor.

4. *The active principle*

Paper towels were cut up with scissors, placed in beakers, and soaked in water, methanol, acetone, and diethyl ether, respectively. After an hour the solvents were decanted and filtered. Discs of Whatman's filter paper were dipped into each extract and then dried. The impregnated papers were placed in Petri dishes along with seven freshly moulted, fifth instar *Pyrrhocoris* larvae.

Five days later, all the larvae exposed to the methanol, acetone, or ether extracts moulted into sixth instar larvae. By contrast, the larvae exposed to the aqueous extract transformed into normal adults after a total of seven days.

The solvent was evaporated from the acetone extract to obtain a crude preparation of the material. The latter was heated in a boiling water bath for 10 minutes, redissolved in acetone, and impregnated onto discs of filter paper for testing. Full activity was retained, thereby demonstrating that the active material is heat-stable.

Another preparation of active extract was dissolved in 2 ml of methanol, mixed with 1 ml 50% KOH, and saponified for 15 minutes at 50° C. After the extraction of the non-saponifiable fraction with ether, the mixture was acidified and the saponifiable fraction extracted with ether. Both fractions were inactive when impregnated onto filter paper and assayed in the usual manner. This shows that the active factor is destroyed by treatment with alkali.

5. *Partial purification of the crude extract*

A hundred towels (*Scott, Brand 150*) were extracted with a total of 4 liters of absolute methanol. The extract was decanted, filtered, and the solvent evaporated on a rotary evaporator; wt 2.32 g.

The crude material was extracted with four 25-ml changes (hereafter, 4 × 25-ml) of petroleum ether (bp 30–60° C). The solvent was decanted and evaporated on a warm plate to yield a golden oil along with a greenish precipitate; wt 1.42 g. The oil solidified on cooling.

The material insoluble in petroleum ether was extracted with 4 × 25 ml benzene to obtain a green immobile film; wt 0.14 g. Further extraction with 4 × 25 ml diethyl ether gave an unweighable white film. Final extraction with 4 × 25 ml absolute methanol provided a pale yellow, amorphous material; wt 0.65 g.

Each of the four fractions was dissolved in acetone, impregnated onto filter paper, and assayed on *Pyrrhocoris*. All the activity was recovered in the initial petroleum ether fraction.

6. *Silicic acid chromatography*

The petroleum ether fraction was dissolved in 100 ml petroleum ether (bp 30–60° C) and filtered to get rid of the insoluble, green sediment. About 60 ml of this solution was percolated onto a 2-cm (ID) column composed of a slurry of 30 g silicic acid ("Unisil") prepared in petroleum ether. The column was eluted successively with 125 ml of petroleum ether; petroleum ether-benzene 1:1; pure benzene; benzene-diethyl ether 1:1; pure ether; and absolute methanol. Assays of the several fractions showed that nearly all the activity was recovered in the fraction eluted in equal parts of benzene and diethyl ether; wt 0.28 g.

Solvent extraction and chromatography therefore achieved an approximately four-fold purification of the original, highly active methanolic extract.

7. *Botanical sources of the active material*

The presence of the active substance in so many American paper products suggested that the factor was derived from one or more species of American pulp trees.

Through the kindness of Professor Irving W. Bailey, authentic samples of seven species of gymnosperms were obtained from the wood collection of the Harvard Herbarium (where the specimens had been stored for 25 or more years). Each specimen was first rinsed in acetone and then cut up on a drill press with an acetone-washed drill. The pulverata were shaken with acetone, filtered, and the solvent evaporated to obtain the acetone-soluble materials. The latter were dissolved in small volumes of methanol, impregnated onto filter paper, and assayed on *Pyrrhocoris*.

High activity was recorded for extracts of balsam fir (*Abies balsamea*), hemlock (*Tsuga canadensis*), yew (*Taxus brevifolia*), and American larch (*Larix laricina*). Extracts of the southern pine (*Pinus echinata*) and European larch (*Larix decidua*) showed only traces of activity, while extracts of red spruce (*Picea rubra*) appeared to be inactive.

These findings point to botanical origins of the active material. Evidently, the substantial activity in American paper products is mainly derived from balsam fir (*Abies balsamea*)—a principal pulp tree indigenous to America. The negative tests recorded for paper samples of European and Japanese manufacture suggest that the pulp trees of Europe and Japan do not contain the active principle.

8. *The entry of the active material into the insect*

Freshly moulted, fifth instar larvae, together with food and water, were placed in a glass jar and the latter capped with copper netting. This jar was placed in a sealed one-gallon jar containing a number of paper towels.

In the absence of any contact with the towelling, all individuals transformed into normal adults. This showed that the active factor is not volatile at room temperature and that actual contact with the factor is necessary.

We supposed that the point-of-entry might be the thin, elastic cuticle of the pulvillus at the tip of each leg. However, when all of the pulvilli were amputated, the bugs were no less sensitive to the towelling.

On close inspection, we found that all individuals touched the paper with their antennae from time to time. Therefore, it seemed possible that the antennae were the points-of-entry. To test this possibility, a piece of copper netting was placed on the towelling and a second netting supported 1 mm above the first. Freshly moulted fifth instar larvae were reared on the second netting so that they could touch the paper with the antennae but not with the legs. All these individuals showed a positive response by moulting into adultoid forms.

In this case the site of entry was, unquestionably, the antennae. However, in other experiments we found that when the antennae were amputated, the larvae remained fully sensitive to direct contact with the towelling.

All these observations suggest that the active material can gain entry through any part of the integument. This hypothesis was examined as soon as the active material was available as an extract.

By means of a micro-syringe, a droplet (*ca.* 0.1 μl) of an acetone solution of partially purified extract was applied to specific body regions in each of a large series of freshly moulted fifth instar larvae. The extract proved to be fully effective when placed on any part of the larva—even at the tip of a leg or antenna. This demonstrated that the active material readily penetrates the intact cuticle and is then translocated throughout the body.

The extract was also fully effective when injected. This was true even when precautions were taken to avoid any contact between the extract and the outside of the cuticle or epidermis.

When diluted with acetone, the extract remained fully effective when topically applied. However, when dissolved in peanut oil, the effects were more or less localized to the site of application.

9. *Experiments on allatectomized larvae*

The juvenile hormone activity of the extract was documented in a direct manner in experiments performed on 17 larvae from which the corpora allata had been removed at the outset of the fifth instar. Each individual then received a

TABLE II

*Effects of contact with partially purified extract impregnated on filter paper**

Purified extract on filter paper (mg)	Number of individuals	5th instar larvae moulted into:
1.0	7	Perfect 6th instar larvae
0.5	7	Perfect 6th instar larvae
0.25	7	Nearly perfect 6th instar larvae
0.125	6	Adultoid 6th instar larvae
0.062	7	Adultoid 6th instar larvae

* Each disc of filter paper was 9 cm in diameter.

topical application of purified extract. Five days later, all individuals moulted into sixth instar larvae or adultoid forms. In each of the 17 individuals, the absence of corpora allata was confirmed by dissection. These findings demonstrated that the active principle is, in fact, a potent analogue of the juvenile hormone of *Pyrrhocoris apterus.*

10. *Quantitative studies*

A weighed sample of the partially purified extract described in Section 6 was serially diluted in acetone and used to impregnate five 9-cm discs of Whatman's No. 1 filter paper with a known amount of extract. Each disc was placed in a Petri dish along with 6 or 7 freshly moulted, fifth instar larvae.

The results are summarized in Table II. After five days all individuals moulted into sixth instar larvae or adultoid forms. Perfect sixth instar larvae were obtained when the paper contained as little as 500 μg of extract. The lowest dose tested (62 μg) was still effective in causing the formation of adultoid forms.

In order to appraise the absolute sensitivity of the bugs, a measured microdrop of the acetone solution was applied to the dorsum of each of 36 freshly moulted, fifth instar larvae. The results, summarized in Table III, show that the lowest dose tested (0.01 μg) caused all individuals to moult into adultoid forms.

11. *Effects on other species*

We were astonished to find that our most active extracts were without any detectable effects when injected into previously chilled pupae of the *polyphemus* silkworm—even when tested in the ultra-sensitive wax-wound assay for juvenile

TABLE III

Effects of topical application of purified extract to 5th instar Pyrrhocoris

Amount applied (μg)	Number of individuals	5th instar larvae moulted into:
10	10	Perfect 6th instar larvae
1	7	Perfect 6th instar larvae
0.1	10	Adultoid 6th instar larvae
0.01	9	Adultoid 6th instar larvae

hormone (Schneiderman and Gilbert, 1958; Williams and Law, 1965). The same negative results were obtained on all the other silkworm pupae available to us: *Hyalophora cecropia, H. gloveri, Antheraea mylitta,* and *Samia cynthia.*

Even more surprising was the finding that the extract was without any effects on two other species of Heteroptera, *Rhodnius prolixus* and *Oncopeltus fasciatus.* These species were also insensitive to prolonged contact with towelling or with filter paper impregnated with active extract.

Additional experiments have demonstrated that few species are sensitive to the towel extract. Indeed, until recently, the only sensitive species had proved to be *Pyrrhocoris apterus,* itself.

12. *Relation of the extract to juvenile hormone*

The extract, as we have seen, shows high juvenile hormone activity when tested on *Pyrrhocoris* and no activity when assayed on *Oncopeltus.* This strange finding was further explored by transplanting active, adult corpora allata into freshly moulted fifth instar larvae of the two species. The adult glands of *Oncopeltus* were fully effective in blocking the metamorphosis of *Pyrrhocoris;* so, also, the adult corpora allata of *Pyrrhocoris* blocked the metamorphosis of *Oncopeltus.*

From these experiments we learn that juvenile hormone is completely interchangeable between the two species. Despite this fact, the paper extract mimics the juvenile hormone of *Pyrrhocoris,* but not of *Oncopeltus.*

13. *Effects of cecropia juvenile hormone on Pyrrhocoris*

A partially purified preparation of *cecropia* oil was utilized which, as previously described (Williams and Law, 1965), routinely shows marked juvenile hormone activity when 5 mg are injected into pupae of *Antheraea polyphemus* (weight 5 g) or into other saturniids. Fifth instar *Pyrrhocoris* weigh 15 to 45 mg and are, therefore, only 1% to 3% the mass of the silkworm pupae. So, if *Pyrrhocoris* is fully sensitive to the juvenile hormone of *cecropia,* the effective dose should be about 0.1 mg.

Five times this dose was injected into each of a series of freshly moulted, fifth instar *Pyrrhocoris* larvae. The hormone had no detectable effects and all individuals moulted into normal adults.

In another experiment, *Pyrrhocoris* larvae were reared in contact with a disc of filter paper impregnated with 25 mg of *cecropia* extract. All individuals underwent normal metamorphosis.

These findings confirm previous observations that the juvenile hormone of the *cecropia* silkworm has little or no effects on *Pyrrhocoris* (Sláma, 1961; 1962). And yet, strange to say, the *cecropia* hormone is effective when injected (Williams, 1956) or topically applied (Wigglesworth, 1958) to *Rhodnius.*

DISCUSSION

1. *The relation of the paper factor and juvenile hormone*

As summarized in Table IV, *Pyrrhocoris* is sensitive, not only to the juvenile hormone of *Oncopeltus,* but also to the paper factor. *Oncopeltus* is sensitive to the juvenile hormone of *Pyrrhocoris,* but insensitive to the paper factor. So, in terms of its inactivity for *Oncopeltus*, the paper factor fails to duplicate all the biological effects of *Pyrrhocoris* hormone. This suggests that, despite its high activity for *Pyrrhocoris*, the paper factor is a different molecule from the authentic juvenile hormone of *Pyrrhocoris apterus*.

2. *The relation of farnesol and juvenile hormone*

Table IV also illustrates the marked differences encountered when the terpenoid alcohol, farnesol, is assayed on diverse species. For certain species, such as *Tenebrio* (Schmialek, 1961), farnesol possesses high juvenile activity. But in assays on many other species—for example, *Pyrrhocoris*, *Oncopeltus*, *H. cecropia*, and *A. polyphemus*—farnesol shows little or no activity. This demonstrates that farnesol can mimic the juvenile hormone of certain species but not of others.

TABLE IV

Sensitivity of five species to juvenile hormones and mimetic substances

Substance	Species of insect				
	Pyrrhocoris	*Oncopeltus*	*Rhodnius*	*A. polyphemus*	*H. cecropia*
Pyrrhocoris hormone	+++++	+++++	?	?	?
Oncopeltus hormone	+++++	+++++	?	?	?
Cecropia hormone	+?	+?	+++	+++++	+++++
Farnesol	+	+	+++	+	+
Paper extract	+++++	0	0	0	0

3. *The relation of cecropia extract and juvenile hormone*

Attention is now directed to the juvenile hormone activity of extracts prepared from the abdomens of male *cecropia* moths. The crude extract (Williams, 1956; Gilbert and Schneiderman, 1960), as well as purified fractions (Schneiderman and Gilbert, 1964; Williams and Law, 1965), show high activity in "pupal assays" performed on *cecropia*, *polyphemus*, and *cynthia*.. But, as indicated in Table IV, little or no activity is evident in tests performed on *Pyrrhocoris* or *Oncopeltus*. This implies that the *cecropia* hormone is chemically different from *Pyrrhocoris* or *Oncopeltus* hormone.

4. *Chemical diversification of juvenile hormone*

All these observations argue in favor of the existence of more than one molecular species of juvenile hormone. Evidently, during the millions of years of insect evolution, the detailed chemistry of the hormone has also evolved and diversified. So, today, there appears to be a number of related molecules—presumably terpenoid in character—which serve as juvenile hormone for diverse insects.

5. *Diversification of the receptor mechanisms*

Evolutionary changes in the chemistry of juvenile hormone would, of necessity, proceed hand-in-hand with homologous changes in the receptor mechanisms in the cells and tissues. It seems altogether likely that this biochemical "retuning" of the target organs includes steric changes in the hypothetical "receptor sites" to accommodate the altered conformation of the hormone.

The probable significance of these changes in the target organs may be illustrated in the case of insects injected with farnesol. In a highly sensitive species such as *Tenebrio*, the detailed properties of the cellular mechanism permit the type of binding prerequisite for full endocrine activity. Presumably, in the case of a less sensitive species such as *Rhodnius,* the binding is less effective. And in the case of insects insensitive to farnesol, one may conjecture that the molecule is excluded from effective binding to the hormone receptors.

6. *The paper factor as an analogue of Pyrrhocoris hormone*

As reported in the section on Results, two lines of evidence suggest that the paper factor acts directly on the tissues of *Pyrrhocoris.* Thus, as we have seen, the extract is fully effective when assayed on allatectomized larvae. Moreover, when the extract was dissolved in peanut oil and topically applied, its juvenilizing effects remained more or less localized.

For these several reasons, the paper factor appears to be a potent analogue of *Pyrrhocoris* hormone which acts directly on the target tissues to provoke the same reactions as the native hormone, itself.

7. *The insensitivity of Rhodnius and Oncopeltus to the paper factor*

We have emphasized that, up to the present time, few species have been found sensitive to the paper extract. The insensitivity of the saturniids (Table IV) is not difficult to comprehend since the separate evolutions of the Lepidoptera and Heteroptera have been going on for at least 200 million years (Prof. F. M. Carpenter, personal communication). The paradox is the insensitivity of *Rhodnius* and *Oncopeltus*—two other species of Heteroptera—to the paper factor.

One possibility is that *Rhodnius, Oncopeltus,* and most other insects possess a mechanism for metabolizing or inactivating the paper factor; for obvious reasons, this mechanism would either be absent or ineffective in the case of *Pyrrhocoris.* In a subsequent communication, we shall present evidence which strongly argues against this hypothesis.

A second possibility has already been considered in connection with the differential sensitivity to farnesol—namely, that in the vast majority of insects the conformations of the receptor sites for juvenile hormone exclude the paper factor from the type of effective, catalytic binding which it promptly achieves with the target organs of *Pyrrhocoris.* We have a high regard for this theory.

A further possibility is that the hormonal receptors are intracellular and that the paper factor is able to enter the cells of *Pyrrhocoris,* but, for some unexplained reason, is unable to enter the cells of *Oncopeltus.* We are unenthusiastic about this proposition.

A final possibility is that the paper factor is selectively bound, but is inactive in *Oncopeltus* and other insensitive species. Under this circumstance the paper factor might be a potent inhibitor of the juvenile hormone. We have been unable to demonstrate any such effects.

A decision among these several hypotheses seems so important that the entire phenomenon is being studied in further detail.

8. *The paper extracts as an insecticide*

The paper factor is little short of a pyrrhocoricide. As reported in the section on Results, the application of 0.01 μg of the partially purified extract is effective in blocking the normal metamorphosis or sexual maturation of fifth instar larvae. One gram of the extract would therefore be sufficient to "kill" 100 million of these bugs on contact.

Chemical studies performed in collaboration with Prof. John H. Law suggest that the partially purified extract contains only a small concentration of the active principle—certainly not in excess of a few per cent. So, the absolute sensitivity of fifth instar *Pyrrhocoris* larvae must be measured in fractions of millimicrograms.

The present investigation has therefore confirmed an earlier suggestion that juvenile hormone is potentially an effective insecticide (Williams, 1956). What was not then appreciated is the apparent diversification, not only of the detailed chemistry of the hormone, itself, but also of the receptor mechanisms at the cellular level. These prospects point the way to the use of selective agents to control selected pests.

Though *Pyrrhocoris apterus* is a benign insect, we have elsewhere (Sláma and Williams, 1965) called attention to the fact that certain other Pyrrhocoridae are major pests, especially in Asia. This we can state with assurance: any species of insect which shares the endocrine sensitivities of *Pyrrhocoris apterus* is subject to control, if not eradication, by the active principle available on an unlimited scale in American paper products.

9. *Insects and the gymnosperms*

All the evidence points to the synthesis of the active factor in the paper extract by the balsam fir (*Abies balsamea*) and certain other species of gymnosperms. By this particular synthesis, the balsam fir and its allies have achieved full protection against any insect which shares the endocrine sensitivities of *Pyrrhocoris apterus*.

The most intriguing possibility is that the active factor in the balsam fir is a "biochemical memento" of the juvenile hormone of a former natural enemy of these trees—a predator which, for obvious reasons, is extinct, or has shifted its diet to other plants, or has survived in areas where these trees do not occur.

There is reason to believe that the active factor in the paper extract is some sort of terpene derivative; indeed, several compounds of this general type have been found to exhibit detectable juvenile hormone activity for *Pyrrhocoris* (Sláma and Williams, unpublished observations). What may be the significance of the vast number of terpenes and terpenoids which the gymnosperms synthesize for no apparent reason? Is it possible that, in the absence of any dependency on insects for pollination, the gymnosperms have evolved an incredibly sophisticated self-defense against insect predation—a method of insect control which we are just beginning to comprehend?

SUMMARY

1. Materials composed of American paper-pulp contain an extractable, heat-stable lipid which exhibits extremely high juvenile hormone activity when injected or topically applied to the European bug, *Pyrrhocoris apterus.* The active principle in the paper materials is derived from certain species of pulp trees, more particularly the balsam fir, *Abies balsamea.* Larvae exposed to the active material ultimately die without completing metamorphosis or attaining sexual maturity.

2. Despite its extremely high activity for *Pyrrhocoris apterus,* the extract is without any detectable effects on silkworm pupae or most other species of laboratory insects; conversely, juvenile hormone extracts prepared from *cecropia* silkmoths show little or no activity when tested on *Pyrrhocoris.* These findings point to a diversification of the detailed chemistry of juvenile hormone and to corresponding changes in the conformation of the receptor mechanism at the cellular level.

3. The factor extracted from paper materials promises to be an effective agent for the selective control of insect pests which show the same endocrine sensitivities as *Pyrrhocoris apterus.* It is possible that hormonally active factors for other species may be present in other gymnosperms.

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THE JUVENILE HORMONE. VI. EFFECTS OF THE "PAPER FACTOR" ON THE GROWTH AND METAMORPHOSIS OF THE BUG, PYRRHOCORIS APTERUS[1]

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We have already described the presence in American paper products of a highly active analogue of the juvenile hormone of *Pyrrhocoris apterus* (Sláma and Williams, 1965, 1966). Thus, when reared in contact with "active paper," *Pyrrhocoris* larvae undergo one or more supernumerary larval moults and finally die without completing metamorphosis or attaining sexual maturity. This same result was provoked when 1 μg of partially purified "paper factor" (PF) was applied to larvae at the outset of the normal, final (fifth) larval instar (Sláma and Williams, 1966).

Our present purpose is to describe in further detail the effects of PF on the growth, moulting, and metamorphosis of *Pyrrhocoris apterus*.

MATERIALS AND METHODS

1. *Culture of Pyrrhocoris*

All cultures were reared at 25 ± 1° C in an incubator equipped with three 15-watt fluorescent lamps programmed for a daily illumination of 17 hours (Williams and Adkisson, 1964). Distilled water was placed in the bottom of the incubator to maintain a moist atmosphere.

The breeding stocks of adult bugs were reared in round glass jars (9 cm O.D. × 9.4 cm) containing: linden seed (*Tilia cordata*), a pleated rectangle of Whatman's No. 1 filter paper, and a cotton-plugged vial of tap-water; the latter was replaced every two or three days. The jars were capped with cotton netting which was held in place with rubber bands.

At daily intervals, the cultures were inverted to filter the fertilized eggs through the cotton netting. Each group of eggs was placed on a 9-cm disc of Whatman's No. 1 filter paper in a 10-cm Petri dish. As soon as the eggs hatched, linden seed and a vial of water were put in each dish, the latter being replaced every two or three days. The larvae were reared to specific stages and then used in the experiments noted below.

2. *Extraction of PF*

Crude PF was extracted from paper towels (Scott Paper Co., Brand 150) by soaking them in absolute methanol. The solvent was removed on a rotary

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evaporator, and the resulting brown sludge was concentrated by extraction into petroleum ether (b.p. 30–60° C). The solution was filtered and chromatographed on a column of silicic acid, as previously described (Sláma and Williams, 1966). The overall purification achieved by these maneuvers was about four-fold.

Weighed samples of the extract were dissolved in acetone or methanol and dispensed with macro- or micropipettes.

3. *Treatment of Pyrrhocoris with PF*

Groups of larvae, at specific stages of development, were placed in 10-cm Petri dishes, along with linden seed and a vial of water, and exposed to PF in one of three ways: (1) a folded rectangle was cut from Scott towelling and placed in the dish; (2) a 9-cm disc of Whatman's No. 1 filter paper, plus a pleated rectangle cut from the same material, were impregnated with an acetone solution containing a known amount of PF and placed in the dish; or (3) by means of a microsyringe, a measured droplet of PF solution was placed on the abdominal tergum of individual CO_2-anesthetized insects.

TABLE I

Scoring system for evaluating the effects of juvenile hormone and mimetic substances on fifth instar Pyrrhocoris larvae

Score	5th instar moult into:
0	Normal adults.
I	Slightly affected adults; forewings shortened and deformed with larval cuticle at their tips; destruction of hindwings incomplete; abdominal sternum covered with black adult-type cuticle.
II	Adultoids with forewings of larval shape and proportions; hindwing lobes fully preserved; abdomen with black adult-type cuticle and patches of transparent larval-type cuticle through which red pigmented epidermis may be seen.
III	Adultoids with larval wings (as above); about half of abdomen covered by transparent larval-type cuticle through which red pigmented epidermis may be seen.
IV	Nearly perfect 6th instar larvae, except for black patches of adult cuticle scattered over abdominal sternites.
V	Perfect 6th instar larvae.

RESULTS

1. *Quantitative aspects of the Pyrrhocoris assay*

Exposure of early fifth instar larvae to PF duplicated all the effects previously achieved by the implantation of active corpora allata (Sláma, 1962, 1964). In each case the presence of juvenile hormone activity was signaled by a failure of the larvae to transform into normal adults.

In practice, it was possible to subdivide the juvenile hormone reactions into six intensities, ranging from 0 (metamorphosis into normal adults) to V (moulting to sixth stage larvae with full and complete preservation of larval characters). Table I summarizes the external characters used in this scoring system.

2. *Normal development of Pyrrhocoris*

The duration of a particular instar is ordinarily considered to be the elapsed time between successive ecdyses. When *Pyrrhocoris* larvae are cultured at 25° C in the absence of PF, this time is five days for each of the first four instars and seven days for the final (fifth) instar.

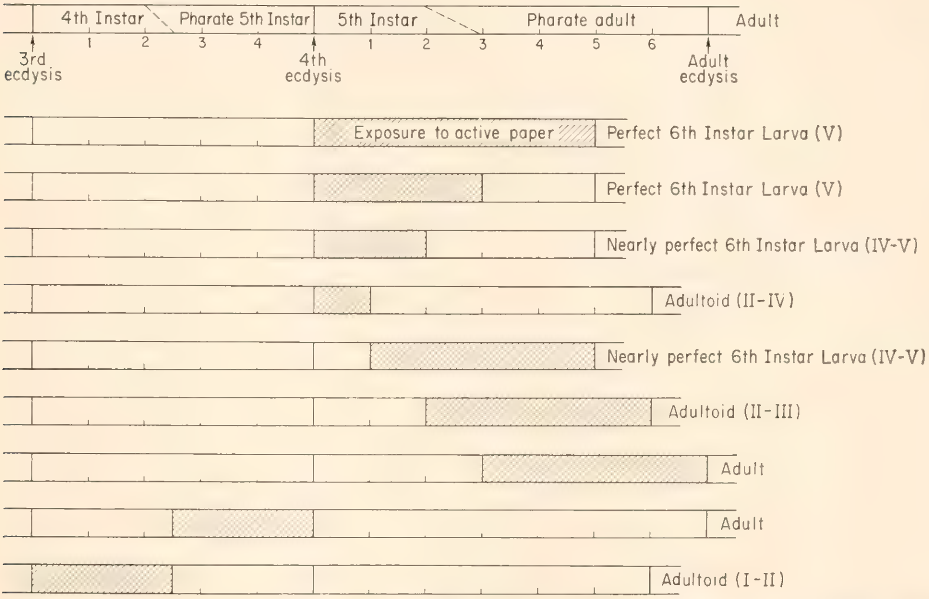
4th Instar
Pharate 5th Instar
5th Instar
Pharate adult
Adult
1
2
3
4
1
2
3
4
5
6
3rd ecdysis
4th ecdysis
Adult ecdysis
Exposure to active paper
Perfect 6th Instar Larva (V)
Perfect 6th Instar Larva (V)
Nearly perfect 6th Instar Larva (IV-V)
Adultoid (II-IV)
Nearly perfect 6th Instar Larva (IV-V)
Adultoid (II-III)
Adult
Adult
Adultoid (I-II)

FIGURE 1. The top diagram illustrates the normal timing of developmental events when fourth instar *Pyrrhocoris* larvae are cultured at 25° C in the absence of PF; as illustrated by the two slanted, broken lines, the pharate stage of the succeeding instars begins two to three days after the third or fourth ecdyses.

The other nine diagrams illustrate the effects of "pulsed" exposure to active towelling during the periods indicated by cross-hatching; the Roman numerals refer to the intensities of the juvenile reactions as scored by the characters noted in Table I. For further details, see text.

As pointed out previously (Sláma, 1964), the epidermal cells, at a certain stage in each instar, detach from the cuticle and retract to form an intervening space which is simultaneously flooded with moulting fluid (Passonneau and Williams, 1954). As Hinton (1958) has emphasized, these happenings signal the beginning of the "pharate" phase of the next instar. In the case of *Pyrrhocoris* larvae cultured at 25° C, the retraction of the epidermis is first initiated in the wing lobes about 48 hours after the preceding ecdysis; the retraction then spreads throughout the body and finally reaches the epidermal cells of the abdomen 1½ to 2 days later. This timing of events is illustrated in the top diagram in Figure 1.

3. *Changes in the sensitivity to PF*

Five hundred larvae were hatched from eggs and subdivided into two equal lots. The control group was reared in contact with inert filter paper; the experimental group, in contact with towelling.

We would emphasize that, during the first four instars, the two groups were indistinguishable. Moulting occurred at five-day intervals and growth proceeded in a completely normal manner. But, at the conclusion of the fifth instar, a conspicuous difference was evident: the control group moulted into normal adults after the customary seven days, whereas all larvae exposed to PF moulted into perfect sixth instar larvae after five days.

This shows that PF is without any immediate influence on larvae possessing active corpora allata and high levels of endogenous juvenile hormone. Sensitivity first becomes evident in the fifth instar—that is, after the animal's own corpora allata have been inactivated and the secretion of juvenile hormone has stopped.

4. *Sensitivity of fifth instar larvae*

The sensitivity of final stage larvae was studied in further detail by placing groups of 6 or 7 individuals in contact with towelling for specific periods. The results, summarized in Figure 1, show that exposure throughout the first three days of the fifth instar was fully effective in blocking metamorphosis and provoking the formation of perfect sixth instar larvae. Exposure begun after the third day was completely ineffective.

Other experiments, summarized in Figure 1, document a declining sensitivity of *Pyrrhocoris* during the first three days of the fifth instar. Sensitivity is maximal during the first day; indeed, it was possible to show that freshly moulted individuals undergo abnormal metamorphosis as a consequence of only one hour of contact with filter paper impregnated with 5 mg PF.

5. *Sensitivity of fourth instar larvae*

As mentioned above, fourth stage larvae are seemingly unaffected by contact with PF. This finding was examined in the experiments diagrammed in the bottom two lines of Figure 1. One group of seven individuals was exposed to towelling during only the first half of the fourth instar; the other group, during only the second half.

As illustrated in Figure 1, exposure during the first half of the fourth instar provoked abnormal metamorphosis at the end of the fifth larval instar. By contrast, exposure limited to the second half of the fourth instar was inconsequential. This surprising result is discussed below.

6. *Stability of PF within the insect*

In testing the stability of PF, we took advantage of the prolongation of development which one can enforce by depriving the larvae of food. Thus, by starving newly moulted larvae, the fifth instar can be prolonged by a corresponding number of days.

In the experiments summarized in Figure 2, four groups of seven individuals received a topical application of 10 μg of PF at the outset of the fifth instar. The groups were then starved for 1, 3, 6, and 10 days, respectively, before being fed.

As diagrammed in Figure 2, PF was sufficiently stable within the insect to permit a three-day prolongation of the fifth instar without any curtailing of the biological effects of the original topical application. When the period of starvation was extended to six days, or to ten days, the effects were progressively diminished. These findings show that PF is slowly metabolized and only gradually inactivated after its entry into the insect.

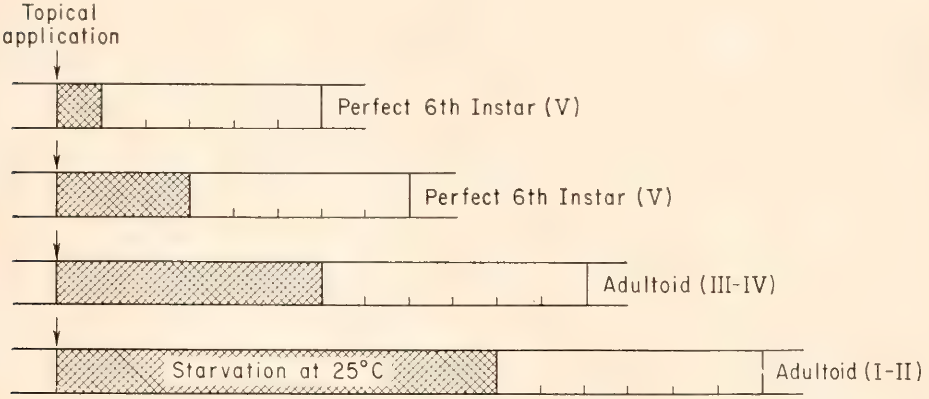

FIGURE 2. The effects of a single topical application of 10 μg of PF extract administered to *Pyrrhocoris* at the outset of the fifth larval stage. In order to prolong the instar, the four groups were starved at 25° C for one to ten days (as indicated by the cross-hatching). The findings document the slow inactivation of PF after its entry into the insect.

DISCUSSION

1. *Juvenile hormone in relation to larval growth and moulting*

Previous studies of *Pyrrhocoris* provide a frame-of-reference for interpreting the juvenile hormone activity of PF. For the most part, these previous investigations have been carried out by investigators in the Department of Insect Physiology in Prague.

By the extirpation, transplantation, and biological assay of living corpora allata, the corpora allata were shown to be active in the secretion of juvenile hormone throughout the first four larval instars (Sláma, 1962, 1964, 1965; Sláma and Hrubešová, 1963). Moreover, during this same 20-day period, the immature larvae were unaffected by the implantation of additional active corpora allata (Novák, personal communication; Sláma, unpublished observations).

In the present study, this result has been duplicated in the finding that continuous exposure to PF is without any detectable effects on growth and moulting during the first four larval instars. Evidently, as long as the titer of endogenous juvenile hormone remains above a certain level, the receptor mechanism at the cellular level is "saturated" and, consequently, insensitive to the introduction of additional hormone.

2. *The critical period during the fifth instar*

Sláma (1964) has demonstrated that the corpora allata become inactive during the moult to the final (fifth) instar. If juvenile hormone is supplied by the implantation of active corpora allata, one can provoke the formation of a supernumerary sixth larval stage provided that the glands are implanted early in the fifth instar. Sensitivity is maximal during the first day after ecdysis and rapidly declines to zero during the first three days (Sláma, 1964). Manifestly, this is exactly the same result as that observed in the present study.

As summarized in Figure 1, we have been able to investigate the changing sensitivity to juvenile hormone with a resolution difficult to achieve by surgical manipulations. Thus, when the exposure of fifth instar larvae to active paper was "pulsed" for periods ranging from one hour to several days, the results were as follows: high sensitivity was documented for each of the first two days, less sensitivity during the third day, and zero sensitivity thereafter. In each region of the body, the loss of sensitivity to PF was synchronized with the activation of the epidermal cells as signaled by their detachment and retraction from the old larval cuticle. These findings are in full accord with those obtained by the implantation of active corpora allata (Sláma, 1964).

3. *Significance of the pharate condition*

As noted in the lowermost diagram in Figure 1, abnormal metamorphosis at the end of the fifth instar was provoked by contact with active paper during the first half of the fourth instar. This documents a remarkable stability of the paper factor once it has entered the insect.

It is therefore paradoxical to find that topical contact with PF was ineffective during the second half of the fourth instar (see next-to-bottom diagram in Figure 2). This result becomes intelligible when one recalls that the larvae are in the pharate condition during the final half of the fourth instar (see topmost diagram in Figure 1). Therefore, in order to gain entry into the body, PF would have to traverse, not only the cuticle and underlying epidermis, but also the intervening layer of aqueous moulting fluid.

Evidently, pharate larvae are resistant to the uptake of PF. This interpretation was tested by *injecting* PF into pharate fourth instar larvae. Of ten individuals subjected to this treatment, all reacted to PF by moulting into adultoid forms at the end of the fifth instar.

4. *Paper factor versus juvenile hormone*

As a physiological analogue of the juvenile hormone of *Pyrrhocoris*, PF appears to be more stable within the insect than is the native hormone itself. In addition to the evidence cited in the preceding section, we have observed that a single topical application at the outset of the fifth larval instar is effective in blocking normal metamorphosis, even when the next moult is postponed for many days

(Fig. 2). By virtue of this stability within the insect, PF as a juvenile hormone analogue shows more long-lasting effects than the native hormone, itself.

Summary

1. American paper products contain an extractable, heat-stable lipid (PF) with high juvenile hormone activity for the bug, *Pyrrhocoris apterus.* When reared in contact with "active paper" or extracts prepared from active paper, larval *Pyrrhocoris* undergo one or more supernumerary larval moults and finally die without completing metamorphosis or attaining sexual maturity.

2. As a potent analogue of the juvenile hormone of *Pyrrhocoris,* PF blocks those aspects of development which can take only in the absence of juvenile hormone—namely, the transformation of the fifth stage larva into an adult. By contrast, it fails to interfere in any way with normal growth and moulting during the first four larval instars—*i.e.,* with developmental events which normally take place in the presence of high levels of endogenous juvenile hormone.

3. Sensitivity to PF is maximal at the outset of the final (fifth) larval instar. At this particular stage, metamorphosis is blocked by contact with PF for a period as brief as one hour. High sensitivity persists during the first two days of the fifth instar and then declines to zero after the third day. In each region of the body, the loss of sensitivity to PF is synchronized with the activation of the epidermis, as signaled by its detachment and retraction from the old larval cuticle.

4. Several lines of evidence suggest that, after its entry into the larval insect, the paper factor is more stable, and therefore more long-lasting in its effects, than is the authentic juvenile hormone of *Pyrrhocoris.*

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CONTROL OF A SEX-LIMITED HAEMOLYMPH PROTEIN BY CORPORA ALLATA DURING OVARIAN DEVELOPMENT IN PERIPLANETA AMERICANA (L.)

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There is considerable evidence to suggest that in a wide variety of insects corpora allata are involved in yolk formation, egg maturation and functional activity of accessory sex glands. The control of yolk formation and egg maturation by corpora allata was first demonstrated by Wigglesworth (1936) in *Rhodnius prolixus* and subsequently has been confirmed in other insects (see Johansson, 1958). In some insects, such as *Calliphora erythrocephala* (Thomsen, 1952) and *Schistocerca gregaria* (Highnam, 1962), it appears that egg maturation and yolk deposition are largely under the hormonal control of the median neurosecretory cells of the brain. In certain insects there is evidence to suggest that corpora allata are involved in various phases of protein metabolism and that the effects on egg maturation and yolk deposition could be due to their influence on protein metabolism (Gilbert and Schneiderman, 1961; Vanderberg, 1963).

The present study is an attempt to test the relationship between corpora allata and protein synthesis during ovarian development in *Periplaneta americana,* and to correlate any changes in protein metabolism with yolk deposition and egg maturation. Haemolymph was chosen as a likely medium to reflect changes in protein metabolism.

MATERIALS AND METHODS

Periplaneta americana adults were taken from a stock colony maintained in the laboratory on dog biscuits and water. The stock colony was started from a larger colony which had been maintained for more than ten years by the Department of Entomology, University of Florida. Newly molted adult females were separated daily and kept separately for surgical procedures. Allatectomy was performed in all cases on females no later than 48 hours after imaginal molt. The allatectomized females and sham-operated controls of the same age were kept with males, for Griffith and Tauber (1942) reported that mating influences egg maturation in *Periplaneta* during the first pre-oviposition period.

Allatectomy

Surgical procedure for removal of corpora allata was that described by Bodenstein (1953). Corpora allata were removed with a pair of finely tipped forceps by severing the allatal nerves without damaging the corpora cardiaca. The pulled-out corpora allata were later examined microscopically to insure complete removal. After the operation a few crystals of streptomycin sulfate were added to the wound and the flap of cuticle pulled back into place and sealed with molten paraffin. Mortality was 15%. The corpora allata of sham-operated controls of the same age as experimental animals were exposed but not removed. All operated animals were able to move about and feed in the normal way.

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Ovariectomy

Ovaries were removed from 7-day-old adults. A roach was anaesthetized with ether and the adbomen placed ventral side down into a groove in a wax plate and firmly held in position with a strip of "plasti-Tak." A small transverse incision was made on the intersegmental membrane between the sixth and seventh abdominal segments. Through the slit the ovaries were removed with a pair of forceps. In controls the same procedure was followed except that ovaries were not actually removed. The wound was sealed with molten paraffin, and unless damage to the gut occurred, the insects made a quick recovery after the operation and fed in a normal manner.

Collection of haemolymph

Haemolymph for electrophoresis and protein determinations was collected by the centrifugation technique described by Siakotas (1960) with the modification that the alimentary canal was not removed from the animals, but mouth and anus were sealed with molten paraffin. Haemolymph was used immediately after collection or stored at −18° C. for future use.

Electrophoretic procedures

Electrophoretic separation of haemolymph proteins was carried out on cellulose acetate strips in a Shandon Universal model electrophoresis tank with a Beckman Duostat power supply. The buffer system, freshly prepared each week, was that of Brackenbridge (1960). Cellulose acetate strips, 2.5 × 12 cm. (obtained from Consolidated Laboratories Inc., Chicago Heights, Illinois), were processed as suggested by Brackenbridge (1960) and placed in position in the electrophoresis tank. Three hundred milliliters of buffer solution were poured into each of the electrode wells. Whatman 3 MM wicks, saturated with the buffer, connected the buffer solution in the electrode wells with the strips. With a calibrated micropipette 2.0 μl. of the centrifuged haemolymph were applied to the center of the strip in the tank. A constant current of 0.4 milliamperes per cm. width of strip was used in all cases. An initial potential of 200 volts fell to 150 volts at the end of a two-hour run, at which time the strips were taken out and dried in an oven at 100° C. for 15 minutes.

Strips were stained with 0.002% Nigrosin in 2% acetic acid for 15–20 minutes, rinsed in water, dried at room temperature and stored in the dark until scanning. Strips were stained for glycoproteins using periodic acid-Schiff reagent as described by Bodman (1957). Stained strips were cleared in paraffin oil and scanned in a model 52-C densitometer (Photovolt Corporation, New York).

Quantitative determination of total haemolymph proteins

Total haemolymph protein concentration was measured by the colorimetric method of Lowry *et al.* (1951). Haemolymph protein determinations were made simultaneously on both allatectomized and sham-operated females of the same age. After sample color development, optical density was measured at 700 mμ against a water blank and a crystalline bovine serum albumen standard.

Radioisotope experiments

A sample of 14 allatectomized and 14 sham-operated females was used for these experiments 2–4 days after operation. Each female received 5 μc. of tritium-labelled amino acids from a mixture of equal parts of glycine, leucine, and histidine (specific activity 2.5 mc./m*M*) in 0.1 ml. of physiological saline. Injection was accomplished through the femoral membrane of the metathoracic legs following anesthetization with CO_2. At intervals of 1.5, 3, 6, 12, 24 and 48 hours after injection, haemolymph was removed and protein precipitated with 95% ethanol to remove all radioactive amino acids not incorporated into the haemolymph proteins. The precipitate was dried, weighed, and samples converted to a uniform condition for counting by a modification of the Schoniger combustion technique reported by Buyske *et al.* (1961) and Oliverio *et al.* (1962). The samples were ignited in a Thomas-Ogg safety oxygen flask igniter (Arthur Thomas Co., Philadelphia, Pennsylvania), whereby tritium was converted to water. A 15.0-ml. sample of toluene-phosphor solution containing 20% ethanol was added to the flask and a 10.0-ml. sample withdrawn for counting in a transistorized Tricarb Liquid Scintillation Counter (Packard Instrument Co.). The result of triplicate 10-minute counts of each sample is presented as specific activity (counts/minute/mg. protein).

RESULTS

1. *Effects of allatectomy on ovarian development*

In all roaches allatectomized within 48 hours after imaginal molt, the ovary remained small and the oocytes neither grew nor deposited yolk. The sham-operated controls laid eggs 15–20 days after the operation. Allatectomy performed on females later than 48 hours after the imaginal molt did not completely prevent egg maturation.

2. *Effects of allatectomy on the electrophoretic pattern of haemolymph proteins*

A typical electrophoretic pattern of haemolymph proteins of allatectomized and sham-operated females at intervals after the operation is shown in Figure 1. The relative amounts of the different fractions of haemolymph proteins, obtained by scanning the strips of Figure 1, are shown in Figure 2. Of the five electrophoretically separable haemolymph protein fractions (arbitrarily numbered 1 through 5), 1–4 migrate toward the anode while 5 migrates toward the cathode. Quantitative analyses of the protein fractions after staining with light green S.F. in potassium biphthalate buffer and subsequently eluting the stains according to the method of Brackenbridge (1960) revealed that in unoperated females, fraction 4 makes up

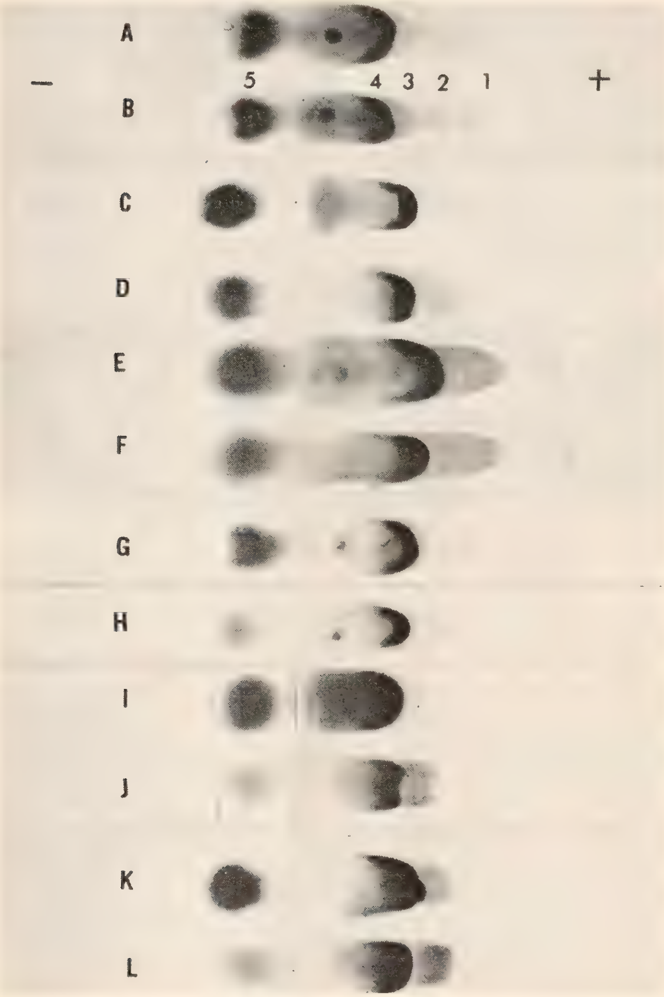

FIGURE 1. Electropherograms of haemolymph proteins of sham-allatectomized (A, C, E, G, I, and K) and allatectomized (B, D, F, H, J, and L) female roaches at respective intervals of 3, 7, 14, 17, 21, and 28 days after the operation. Separated fractions arbitrarily numbered 1–5.

45–51% of the haemolymph proteins, while fraction 5 constitutes 39–45% of the total proteins.

The general results in Figures 1 and 2 have been verified in several electropherograms of allatectomized and sham-operated roaches at each interval after operation. Fraction 5 decreases in concentration after allatectomy, in contrast to a relatively constant concentration in this fraction from the sham-operated females. No significant changes were evident in fraction 4. The other fractions were too small to detect any differences.

The decrease in concentration of protein in fraction 5 observed in the allatectomized females prompted studies to observe the nature of this fraction in the

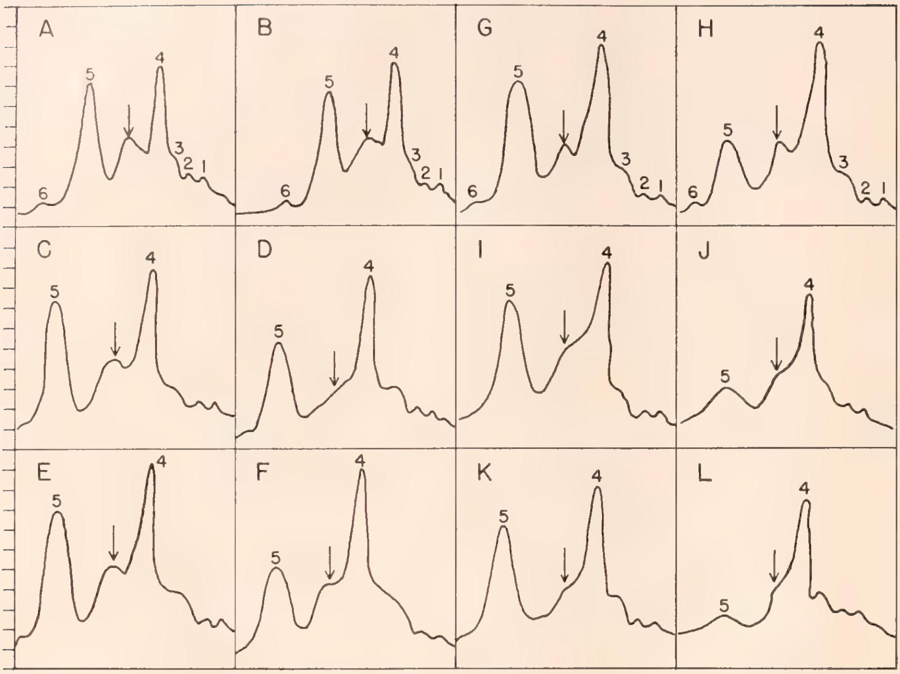

Figure 2. The relative concentrations of haemolymph protein fractions 1–5 from electropherograms of sham-allatectomized (A, C, E, G, I, and K)and allatectomized (B, D, F, H, J, and L) female roaches at respective intervals of 3, 7, 14, 17, 21, and 28 days after the operation. Arrow indicates point of application of haemolymph sample.

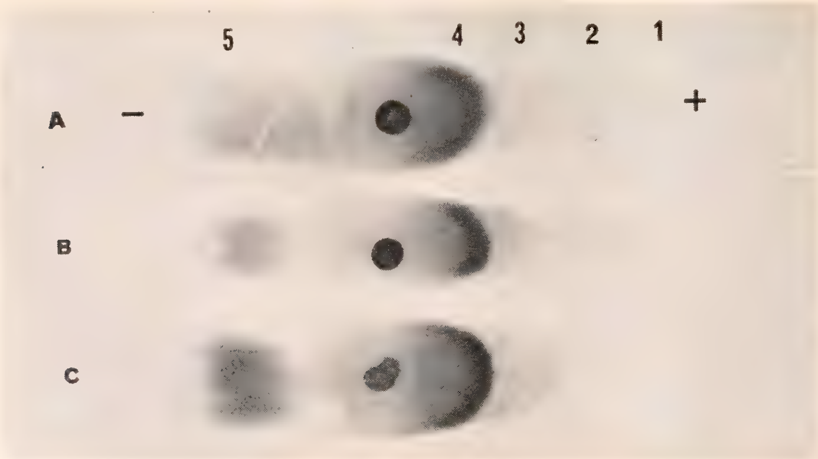

Figure 3. Electropherograms of haemolymph proteins from a sham-allatectomized male (A), allatectomized female (B), and sham-allatectomized female (C) roach 14 days after the operation.

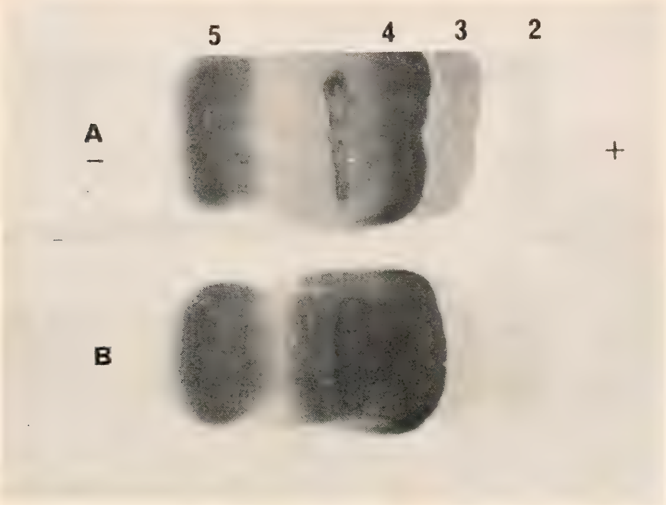

FIGURE 4. Electropherograms of haemolymph proteins from sham-ovariectomized (A) and ovariectomized (B) roaches 7 days after the operation.

male. A typical electrophoretic pattern of the male haemolymph proteins is shown in Figure 3. Protein of fraction 5 from the male is low in concentration compared to that from the female of the same age. This suggests that fraction 5 may be sex-linked, being present in greater concentration in the female than in the male. To test this further, electrophoretic studies were made on haemolymph of ovariectomized females, and a typical electrophoretic pattern obtained 7 days after ovariectomy, along with that of a sham-ovariectomized female, is shown in Figure 4. Protein in fraction 5 has increased in concentration in the ovariectomized roach in excess of that in the sham-operated control. Fractions 4 and 5 in ovariectomized roaches showed additional changes in composition during aging. After the initial

TABLE I

Comparison of haemolymph protein concentrations of allatectomized and sham-operated control females of the American cockroach, Periplaneta americana

Days after operation*	Allatectomized		Sham-operated control	
	Number of females	Haemolymph protein concentration (g./100 ml.) $\bar{X}$ ± SD	Number of females	Haemolymph protein concentration (g./100 ml.) $\bar{X}$ ± SD
7**	5	1.47 ± 0.07	5	1.86 ± 0.13
14	5	1.41 ± 0.16	5	2.03 ± 0.25
21	5	1.29 ± 0.08	5	1.75 ± 0.09
28	5	1.25 ± 0.13	6	1.85 ± 0.14
35	5	1.09 ± 0.14	6	1.71 ± 0.10

* All insects operated upon 8–48 hours after imaginal molt.

** Five newly molted females had an average concentration of 0.85 ± 0.02 g./100 ml. haemolymph.

TABLE II

Comparison of haemolymph protein concentration of ovariectomized and sham-operated females of the American cockroach, Periplaneta americana

Ovariectomized		Sham-operated	
Number of females	Haemolymph protein concentration (g./100 ml.) $\bar{X} \pm SD$	Number of females	Haemolymph protein concentration (g./100 ml.) $\bar{X} \pm SD$
4	2.59 ± 0.18	5	1.82 ± 0.13

All insects operated upon 7 days after imaginal molt.
All protein determinations were made 14 days after operation.

increase in stainable material following ovariectomy, fraction 5 began to decrease after about 14 days in stainable material, and after 30 days very little protein was left. In the meantime fraction 4 began to increase in material which stained as glycoprotein.

3. *Effects of allatectomy and ovariectomy on total haemolymph protein concentration*

The total haemolymph protein concentration of allatectomized and sham-operated females is shown in Table I. Total haemolymph protein concentration of allatectomized females decreases far below that of sham-operated controls.

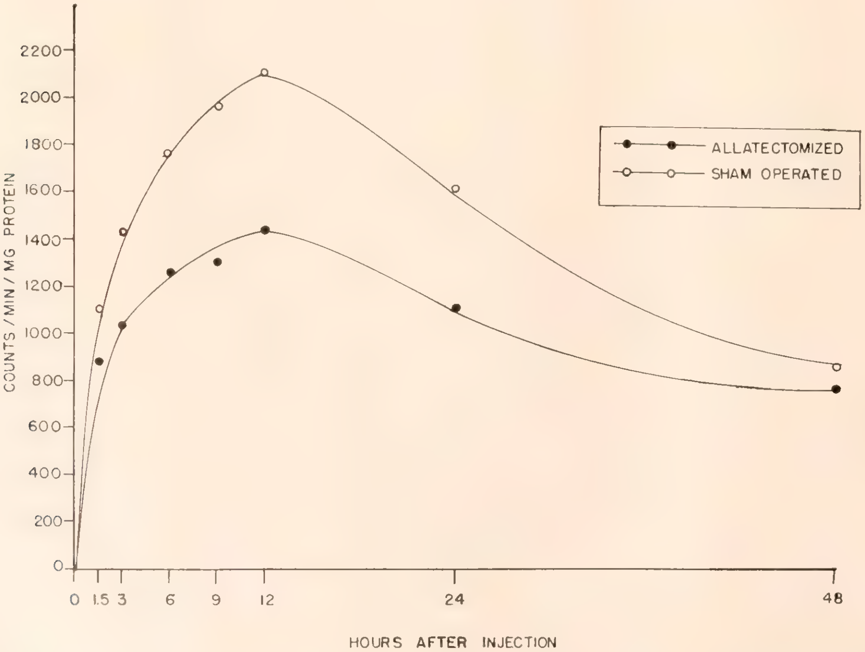

FIGURE 5. Comparison of incorporation of tritium-labelled glycine, leucine, and histidine into the haemolymph proteins of allatectomized and sham-allatectomized female roaches (5 μc./roach). Points are averages of two experiments.

Haemolymph protein concentrations of 14 days post-ovariectomized females and of sham-ovariectomized females of the same age are shown in Table II. It is clear that an increase in blood protein concentration follows ovariectomy.

4. *Effects of allatectomy on incorporation of H^3-labelled amino acids into haemolymph proteins*

Figure 5 shows that allatectomy inhibits incorporation of H^3-labelled amino acids into haemolymph proteins. The maximum rate of incorporation occurred 12 hours after injection of the amino acids, at which time the controls exhibited a rate which was 1½ times that of allatectomized females. We have no evidence that labelled proteins were synthesized in the haemolymph; they may have been synthesized elsewhere and then released into the haemolymph.

Discussion

The interactions of the various hormones involved in insect reproduction are not fully understood. The role of corpora allata in egg maturation and in the functional activity of the accessory sex glands has been well established in all the major orders except Lepidoptera (see Johansson, 1958). Among roaches several species have been studied and the influence of corpora allata on egg maturation confirmed. The present study with *Periplaneta* supports the findings of Scharrer (1946) in *Leucophaea*, Engelmann (1959) in *Diploptera*, and Roth and Stay (1959) in *Blatella*, that allatectomy does interfere with the normal development of the ovaries.

Though endocrine control of egg maturation has been well documented, the nature of the hormonal control is still a matter of dispute. Pfeiffer (1945) has shown in *Melanoplus* that the corpora allata are the source of a metabolic hormone which controls metabolism of fat and mobilization of some non-fatty materials and facilitates the production of materials necessary for yolk deposition. The concept of a metabolic hormone was further strengthened by L'Hélias (1953), who postulated that the hormone from the corpora allata favors synthesis of proteins. Wigglesworth (1954) maintains that deficiency of yolk deposition in the oocytes of *Rhodnius* may be due to decreased protein synthesis. Recently Vanderberg (1963) presented evidence to show that corpora allata are involved in synthesis of proteins and RNA. Highnam *et al.* (1963) argue that oocyte growth in *Schistocerca* is facilitated by an allatum-produced gonadotrophic hormone exerting its influence, not by general metabolic effects, but by mediating the uptake of proteins from the haemolymph by the oocytes. Furthermore, Highnam *et al.* (1963) found that haemolymph protein levels in allatectomized *Schistocerca* adults exceeded the levels found in sham-operated controls.

The failure of the haemolymph protein concentration in allatectomized *Periplaneta* females to reach the level characteristic of sham-operated control females, and the depressed protein synthesis from labeled amino acids in allatectomized females, are interpreted by us to support the data of Pfeiffer (1945), L'Hélias (1953), and Wigglesworth (1954) that allatectomy does interfere with at least certain aspects of protein synthesis and metabolism.

The gradual decline in concentration of protein fraction 5 (Figs. 1 and 2) following allatectomy further suggests that ovarian development in *Periplaneta* is related to a protein or proteins whose synthesis is somehow under control of the corpora allata. Fraction 5 may be sex-limited, for in all males tested fraction 5 stained only weakly, giving much the appearance of allatectomized females after 14 days. Stephen and Steinhauer (1957) and Siakotas (1960) demonstrated by electrophoretic techniques not directly comparable with the technique we have used that sex-limited or sex-related differences in concentrations of protein in separated bands do exist in *Periplaneta* haemolymph. Telfer (1954) not only demonstrated the existence of a sex-limited protein in haemolymph of *Platysamia cecropia* females, but by immunological means showed that this same protein enters the eggs during maturation.

Although we have no data to indicate that fraction 5 protein enters the egg during maturation, ovariectomy does allow an increase in stainable material in fraction 5 for a few days following the operation (Fig. 4), which is certainly suggestive that fraction 5 protein may be removed from the haemolymph by the ovaries. After 14 days post-ovariectomy, however, there is clear evidence that the protein compositions of both fractions 4 and 5 are being altered, with fraction 5 decreasing in stainable material and fraction 4 increasing in stainable material which stains as glycoprotein. These changes have not been observed in control females. We interpret the 14 days post-ovariectomy changes as a failure of the maintenance of normal allatum activity in the absence of the ovaries. Since *Periplaneta* frequently lives beyond the reproductive period, it would be interesting to know if similar changes occur during senescence.

Recently Englemann (1965) has observed a correlation between the haemolymph protein concentration and changes in activity of innervated and isolated corpora allata of *Leucophaea*. He thinks that nutrition acts directly on the corpora allata and not through the mediation of the central nervous system. Protein metabolism in turn is also influenced by the corpora allata. He attributes the hypertrophy of the corpora allata after ovariectomy to a persistently high protein level in the haemolymph. Our results support the observations of Englemann (1965).

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SUMMARY

1. The removal of corpora allata from females of the roach, *Periplaneta americana,* soon after the imaginal molt prevented growth and yolk formation in the oocytes.

2. No immediate effect on ovarian development was noticed when mature adult females were allatectomized.

3. The haemolymph protein concentration of allatectomized females failed to reach the level of the sham-operated female roaches of the same age.

4. Electrophoresis of haemolymph proteins on cellulose acetate strips revealed a gradual decrease and final disappearance of one of the main protein fractions in the allatectomized females.

5. The haemolymph protein concentration of ovariectomized roaches rose to a level far in excess of that of sham-ovariectomized roaches of the same age. A significant increase in protein fraction 5 has been observed in the ovariectomized roaches compared with that of sham-ovariectomized roaches.

6. In male roaches fraction 5 remained at low concentration at all times, suggesting that protein fraction 5 is sex-limited in character.

7. Allatectomized roaches exhibited a slower rate of incorporation of tritium-labelled amino acids into haemolymph proteins than the sham-operated roaches.

8. It is concluded that the effects of corpora allata on egg maturation and yolk deposition may be due to their influence on metabolic processes, particularly the synthesis of a sex-limited haemolymph protein which presumably enters the growing oocytes.

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AERIAL RESPIRATION IN THE LONGJAW MUDSUCKER GILLICHTHYS MIRABILIS (TELEOSTEI: GOBIIDAE)

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Fishes that normally inhabit poorly oxygenated waters are often endowed with some mechanism enabling them to respire aerially (Carter, 1931, 1957; Bertin, 1958; Saxena, 1963). Bertin classified such adaptations as: (1) arborescent and labyrinthiform branchial organs, (2) pharyngeal organs, including the buccopharyngeal cavity and its diverticula, (3) esophageal organs, including the swimbladder and lungs, and (4) intestinal organs. Most reported air-breathers are fresh-water species, usually inhabitants of stagnant tropical swamps where deoxygenation is widespread in the protected, shaded waters. Marine fishes, which inhabit exposed waters subject to continuous wind mixing and oxygenation, generally lack special air-breathing organs (Carter, 1957), although Saxena (1963) recognized the "fishes of the estuaries" as constituting a "main association" of air-breathing species. The Gobiidae and related Blenniidae comprise one of the few groups of primarily marine teleosts that reportedly contain several species with such organs (*cf.* Schöttle, 1931; Oglialoro, 1947; Bertin, 1958). These species, moreover, often inhabit marginal areas such as mangrove swamps or quiet estuaries where characteristically large spatial, diurnal, and seasonal fluctuations of oxygen, depth, *etc.* create for them problems shared with denizens of fresh-water swamps.

The longjaw mudsucker (or simply "mudsucker") *Gillichthys mirabilis* Cooper is one of several gobies that can breathe either aerially or aquatically as the occasion requires. Like other air-breathing gobies, its accessory respiratory organ is buccopharyngeal of Bertin's type 2: at the surface the fish gulps air, which is then held in the highly vascularized buccopharynx for respiratory exchange. Typically it inhabits the shallow muddy backwaters and tidal flats from central and southern California, including the Salton Sea, southward along the Pacific Coast of Baja California to the Gulf of California, where in the northern half it is sympatric with its only congener, *G. seta* (Ginsburg) (Barlow, 1961b, 1963). At low tide *G. mirabilis* lives in small pools or even ". . . above the water level in burrows in the bank" (Barlow, 1961b). In an enclosed, constant-level saline lagoon on the U.C.S.B. campus the fish is continuously submerged but often tolerates almost anoxic conditions. Although it occurs naturally in waters of moderate to high salinity, it can tolerate even soft fresh water for varying periods up to two weeks (Barlow, 1961b; personal communication). Therefore, because its natural environment is unstable, *Gillichthys* must periodically tolerate fluctuating oxygen concentrations, near-anoxic conditions, and emergence.

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Similar adaptations in other gobies, such as the mudskipper *Periophthalmus,* have been carefully described (*e.g.,* Schöttle, 1931). Little has been reported, however, of the specific adaptation of *Gillichthys* to aerial respiration, although Barlow (1961a, 1961b, 1963) thoroughly investigated its taxonomy and geographical variation, both morphological and physiological, and Weisel (1947) as well as Barlow (1961a, 1961b) briefly noted its accessory ability to respire aerially. In the present study, therefore, we shall describe the environmental aspects of its aerial respiration by: (1) analyzing its behavioral adaptation in deoxygenated water of gulping at the surface and retaining air bubbles; (2) demonstrating respiratory gaseous exchange in these bubbles while they are retained; (3) describing the structure of the repository of these bubbles (buccopharyngeal organ), including its morphological adjustment before an episode of gulping; and (4) relating this adjustment to a possible accessory function of the swimbladder.

Materials and Methods

Caught locally in an enclosed lagoon and in a tidal slough, the fish were held in well-aerated running sea water of salinity 33.1–34.0‰ at 16–20° C. in 15-gallon wooden aquariums. At 20° C., dissolved oxygen measured about 7 mg./l., carbon dioxide 2–3 p.p.m. In the lagoon, however, oxygen ranged from 0 mg./l. to supersaturation, while carbon dioxide was commonly 16–25 p.p.m. Experimental fish were studied in controlled oxygen environments maintained in 5-gallon stainless steel aquariums at 20–21° C. All fish were fed mussels, on which they thrived. Low oxygen concentrations were obtained in the experimental aquariums by boiling sea water or bubbling nitrogen through it, which is perhaps preferable in that the organic constituents remain relatively unaltered. Dissolved oxygen was measured with a commercial galvanic type oxygen analyzer (*cf.* Duxbury, 1963).

Aerial respiration

We observed the mudsucker's general behavior leading to gulping air at the water surface among a group of 13 fish. We tallied their progressive reactions to each of 7 different oxygen concentrations, which constituted a stepwise decrease from 2.8 to 1.1 mg./l. In another experiment, fish were immediately placed in deoxygenated water (0.5 mg./l.) to determine the necessary period of adjustment before the initial gulp.

To measure the consumption of aerial oxygen, 4 fish, 117–165 mm. in total length, were put into critically deoxygenated water (less than 1.0 mg./l.). When oxygen concentrations were low enough to apparently preclude aquatic breathing (*i.e.,* opercular movements were undetectable), the fish would periodically rise for atmospheric air. Then by immersing them tail first with their mouths held open, all air was forced from their buccopharynx and they were confined, one at a time, within a partly submerged, water-filled, 6-inch glass funnel, which was inverted in an aquarium. Gulped and expelled air was retained in the apex of this funnel whose exposed, cut-down spout was capped by a rubber diaphragm (Fig. 1). After the water level in the aquarium was adjusted to preclude any volumetric change in it, 3 or 6 ml. of air were injected by hypodermic syringe through the diaphragm into the exposed cap of the funnel, thereby displacing an equal volume of water and registering volumetrically at the apex calibrated in ml. This volume was kept minimal to insure the most accurate possible determination of its percentage oxygen change. For the 117-mm. fish (25.5-g.) the volume injected was 3 ml. For the 140- and 165-mm. fish (30- and 36-g.), however, it was necessarily increased to 6 ml., so that the fish could project their mouths above the surface to gulp in the confined space. Gulping would always occur quickly and the volume of air taken was measured by noting the water displacement in the apex; this volume was retained intact by carefully lifting the funnel over and away from the fish. Only those experimental runs were accepted in which the fish released all of the air held. After the fish had expelled all the retained gas under the funnel, the percentages of oxygen and carbon dioxide in the resulting new volume of gas at the apex were measured with a Scholander microgasometric analyzer (Scholander *et al.,* 1955), after first removing a 2-ml. sample with a 5-ml. syringe.

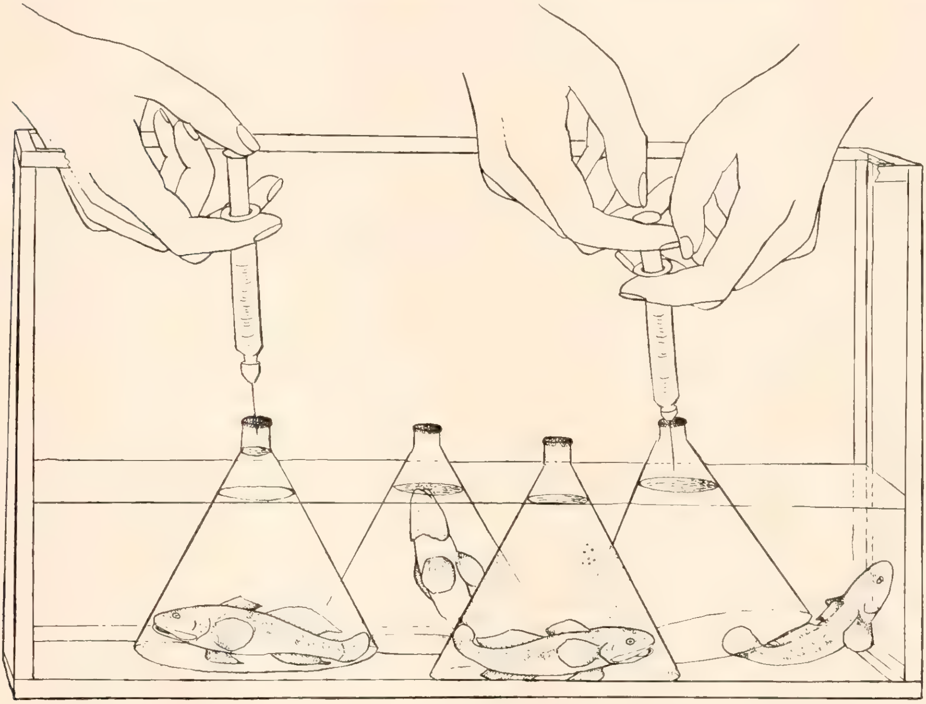


FIGURE 1. Determination of aerial oxygen consumption in *Gillichthys mirabilis.* From left to right: an inoculum of air is provided a gulping fish restrained under a filled funnel in deoxygenated seawater; the fish soon gulps this air in the funnel apex; it returns to the bottom, retaining the bubble but expelling excess air; after a brief sojourn it releases the bubble and is allowed to escape, so that the altered volume of air in the funnel can be analyzed for per cent oxygen and carbon dioxide.

Before each successive observation, all air was again forced from the fish's buccopharynx. During the four runs, each with a different fish and including 7–12 complete observations, we noted the elapsed time between gulping and expelling, *i.e.*, the time that the fish remained submerged with the bubble. Linear regressions were then calculated of the percentage of oxygen remaining in the inoculum of air and of the volume of oxygen extracted from the bubble on time submerged, and of the volume of carbon dioxide expired on that of oxygen consumed.

Volumes were calculated using the reasonable assumption that the original inoculum contained 21% oxygen and 0.03% carbon dioxide; gasometric analyses of air bubbled into the funnel confirmed the negligible alteration of these percentage volumes through the water. The volume of these gases respired between gulping and expelling, therefore, could be approximated from the formulas:

$$V = \frac{I(0.21 + P') - P}{1.0 - P} \quad \text{and} \quad V' = \frac{I(0.03 - P') + PV}{P - 1.0}$$

(noting that calculation of V′ depends on first calculating V), where V equals the volume of oxygen respired, V′ the volume of carbon dioxide expired, I the volume of innoculum (3 or 6 ml.), P the percentage of oxygen in the funnel after expiration, and P′ the percentage of carbon dioxide after expiration. Because it was usually impossible to measure small volumetric changes on the roughly calibrated funnel apex, the calculated volumes respired were generally unsubstantiated by direct observation.

To further investigate the onset and control of gulping, the two larger fish were placed in a 3-gallon all-glass aquarium fitted with a gas-tight lucite lid drilled with a gas inlet, outlet, and a stoppered center aperture for monitoring temperature, salinity, and gas concentration in the water. After the water was deoxygenated (less than 0.5 mg./l.) by bubbling with nitrogen, the fish were placed one at a time under an atmosphere of pure nitrogen maintained in a 2-inch space above the water level. After recording their reactions and allowing them to readjust to normal conditions, they were placed under a 1:1 mixture of nitrogen and carbon dioxide. All gases were piped into the experimental aquarium from adjacent pressure tanks fitted with demand valves. Later, the aquarium cover was removed and the water containing the fish was aerated with the same gases; first nitrogen, then the mixture. We observed the frequency and pattern of surface gulping by each fish under the four conditions.

Following the preceding experiments, in which the air in the funnel was renewed after each gulping sequence, another in which the air was not renewed was undertaken to determine the efficiency of aerial respiration during a continual increase in respiratory carbon dioxide concentration. Oxygen uptake from, and carbon dioxide excretion into the bubble were calculated and the behavior of the subject was noted during the course of the experiment (about 30 minutes) when the fish had access to only the original inoculum of air. Also, the size of gulp was compared with the resulting depletion of oxygen in the funnel apex after the bubble was subsequently released.

To investigate possible differences in rates of oxygen consumption between individuals that are emergent but moist (reported by Barlow, 1961a, for fish breathing pure oxygen) and others that are submergent but gulping air, oxygen uptake was also measured among four fish (all about 90 mm. in total length, weighing 7.45–10.95 g.) in a Mark brand Scholander microvolumetric respirometer. The regular tubular chambers of the apparatus were replaced by 250-ml. reagent bottles with ground glass fittings to hold the half-grown fish, so that they would have ample room in which to move about. A potassium hydroxide boat in each chamber removed excess carbon dioxide during the experimental runs. Following Barlow's procedure, the fish were not fed for several hours before the experiment. Each fish was blotted gently and weighed before each run. Two daylight experiments, each comprising 40 observations (10 observations each among four fish), were carried out at 20 ± .03° C. in a constant-temperature bath: one in moist chambers containing about 5 ml. of unfiltered sea water and atmospheric air, where the fish had to breathe aerially without gulping; the other in chambers half-filled with deoxygenated sea water, where they gulped air at the surface. The fish continued gulping throughout the second experiment, even though the "deoxygenated" water became progressively aerated (from 0.8 to 2.15 mg./l.) as a result of the fish's motion in the chamber. All fish were placed in the respirometer chambers 48 hours before the first measurement, which was subsequently discarded because the fish were hyperactive when first approached. The fish soon adjusted to the experimental regime, however, and subsequent measurements showed no significant "within fish" differences in oxygen consumption among the 15-minute trial periods. These relatively short trials probably caused the fish to be in a continuous state of active metabolism during the entire experiment, which, according to Barlow, implies that their oxygen consumption should be about twice that during a resting state of standard metabolism. After each trial the excursion of the piston indicator of chamber pressure was read, the chamber was flushed with air and reactivated, the fish was allowed a short period of readjustment, and the next trial was initiated. The four fish were observed concurrently during each experiment. Possible differences among trials, animals, and treatments (experiments) were tested in an analysis of variance and the recorded oxygen consumption was compared with rates (cc./kg. body weight/hr.) presented elsewhere.

Structure of the buccopharyngeal organ

Gross dissections were made of the buccopharyngeal region only insofar as its structure is directly concerned with aerial respiration. The extent and development of vascularization in the buccopharyngeal epithelium of fish from deoxygenated water was compared with that of others from aerated water. Also, the buccopharynx was dissected and carefully examined after the fish had been injected intracardially with black India ink. (Half-grown fish were not injected because their integument was relatively transparent.)

Superficial vascularization in *G. mirabilis* was then compared with that of similarly injected specimens of the killifish *Fundulus parvipinnis*, which is an obligatory aquatic breather and locally is sympatric with *G. mirabilis*. The general extent of buccopharyngeal vascularization in the two species was compared after individuals had been placed in deoxygenated water. For a histological comparison, small squares of epithelium were excised from the posterior part of the capillary bed in the buccal roof of each of 6 fish (three of each species) that had been living in poorly oxygenated water and four fish (two of each species) from aerated water. These 10 epithelial squares were fixed in Bouin's solution and two from each group (8 total) were chosen at random to be embedded in paraffin, stained in Mallory's triple stain, and sectioned at 10 μ.

Composition of gas in the swimbladder.

We investigated the possible function of the swimbladder as an auxiliary respiratory organ because: (1) the fish must have an oxygen source during its adjustment period before gulping, and (2) its red gland (gas-secreting and -concentrating organ in the swimbladder) is enlarged relative to that of most other littoral fishes.

First, the swimbladder gas composition of fish in aerated water was compared with that of others in deoxygenated water. Four pairs of adult fish were removed from an aerated aquarium and placed in continuously aerated bowls of sea water. After 15 hours a 0.5-ml. sample of gas was drawn from the swimbladder of one fish of each pair by coelomic puncture with a 5-ml. hypodermic syringe while the fish was held firmly underwater. This was analyzed for the percentages of oxygen and carbon dioxide in the microgasometric analyzer. Meanwhile, the second fish were placed in deoxygenated water for periods ranging from 0.5–15 hours, after which they were removed for swimbladder gas analyses.

A comparison of swimbladder gas among fish living in differing environments was made by analyzing gas samples from fish in an aerated aquarium, a deoxygenated aquarium, a large circular outdoor concrete tank with stratified layers of different oxygen concentrations, the campus lagoon, and an aquarium under an atmosphere of pure nitrogen. Also, gas content was measured among groups of 5 fish each at 6, 12, 24, and 48 hours after their swimbladders had been emptied by coelomic puncture.

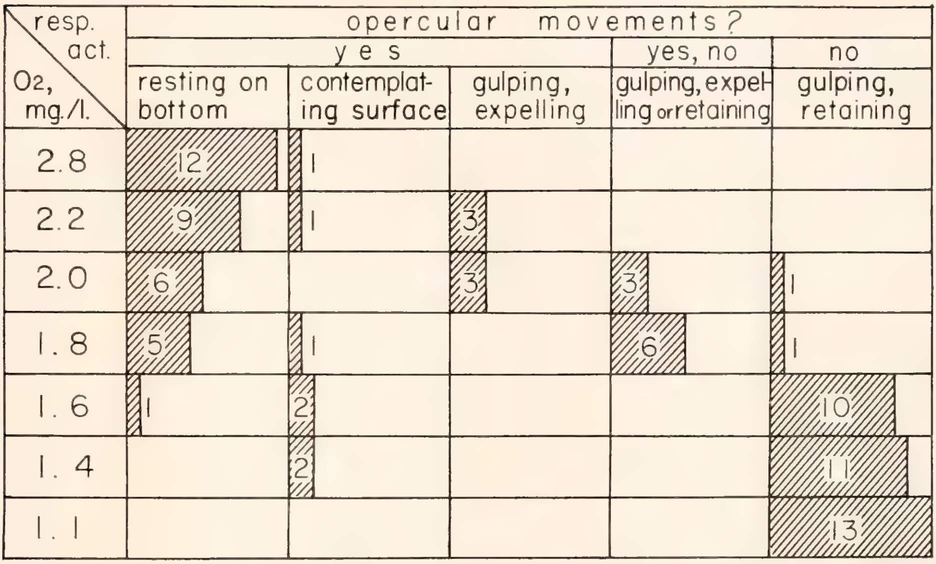
The approximate volume of the swimbladder was measured as the maximum amount of gas removable by a syringe from fish in shallow water.

RESULTS

Behavioral responses to low oxygen concentrations

Our general observations indicate that adult mudsuckers always respond to critically low oxygen concentrations (less than 1.6 mg./l.) by breathing aerially; they cease all opercular movements, gulp mouthfuls of air at the surface, then usually descend for a sojourn on the bottom, and finally release the spent air through the mouth before the next ascent. (Occasionally, in water containing very little oxygen, they remain vertically suspended with snouts contacting the surface.) When denied access to the surface by, for example, a plastic screen, they suffocate when the oxygen concentration is lowered much below the critical level. Young and halfgrown (to 90 mm. total length), however, are facultative gulpers and often continue opercular movements and remain at the surface while apparently breathing aerially. Unlike a typical non-air-breathing fish which usually reacts violently when first removed from water, the mudsucker remains motionless and gulps air at a measured pace. The capacity of its buccopharyngeal cavity can be increased by a strong depression of the tongue and hyoid bones, resulting in a gular expansion of more than 50% of the total volume of the cavity. Like other air-breathing gobies, the fish tends to hold the air anteriorly in this cavity and props its head slightly higher than its tail.

Specifically, when dissolved oxygen fell below 2.0 mg./l. in the experimental aquarium, the cessation of the fish's opercular movements usually preceded the onset of surface gulping by several minutes. Moreover, the rate of opercular movement did not appreciably change as the oxygen concentration was gradually lowered to critical levels: at 5.6 mg./l., opercular beats among 5 aquatic breathers averaged 58.2/min. (range, 48–78); at 4.0 mg./l., 48.5 (40–64) among the same fish; at 2.8 mg./l., 59.5 (38–84); at 2.2 mg./l., 57.5 (40–80) among these fish, one of which was now abortively gulping; at 2.0 mg./l., 53.4 (36–76) among the three fish that were still breathing aquatically; and at 1.8 mg./l., 60.7 (36–76) among these three, two of which were now gulping sporadically. It is emphasized that even when adults began gulping in oxygen concentrations greater than about 1.6 mg./l., opercular movements often continued at the normal rate. Preliminary to the functional gulping, about half of the fish performed abortive intention movements, obviously related to gulping behavior (Fig. 2). Before oxygen had fallen to the critical level, they breathed normally and often rested quietly on the bottom, supported and anchored by their fused pelvic fins, which function as suction cups. As the oxygen was decreased below 3.0 mg./l., however, they would either begin to glance upward, then swim toward but not to the surface, or they would simply approach the surface without the preliminary glancing. Then, as the concentration was further decreased, some fish would break the surface and gulp air, which, however, was almost immediately expelled through the mouth. Finally, at the critical concentration of about 1.6 mg./l., they would assume the normal aerial response of gulping mouthfuls of air, which were held for an average of 3.5 minutes. Interestingly, the other fish either forewent some of the preliminaries or simply assumed the functional gulping response immediately.



resp. act. / O_2, mg./l.	opercular movements?				
	yes			yes, no	no
	resting on bottom	contemplating surface	gulping, expelling	gulping, expelling or retaining	gulping, retaining
2.8	12	1			
2.2	9	1	3		
2.0	6		3	3	1
1.8	5	1		6	1
1.6	1	2			10
1.4		2			11
1.1					13

FIGURE 2. Progressive reactions of 13 mudsuckers to successively lowered dissolved oxygen concentrations. The hatched areas represent proportions of fish exhibiting designated reaction; the numbers in the blocks are the actual counts.

The adult fish that were quickly transferred from aerated to deoxygenated water hesitated 2–58 minutes (usually 15–20) before gulping, during which time opercular movements ceased. Half-grown fish also hesitated for comparable periods, but their opercular movements continued longer. While gulping, several fish floated almost vertically at the surface with their snouts above water; this behavior was repeated on several occasions.

Consumption of aerial oxygen

Expectedly, aerial oxygen was consumed in almost direct proportion to the duration of bubble retention, which varied from 0.83–10 minutes during the four experimental runs and was apparently dependent on the volume of air carried to

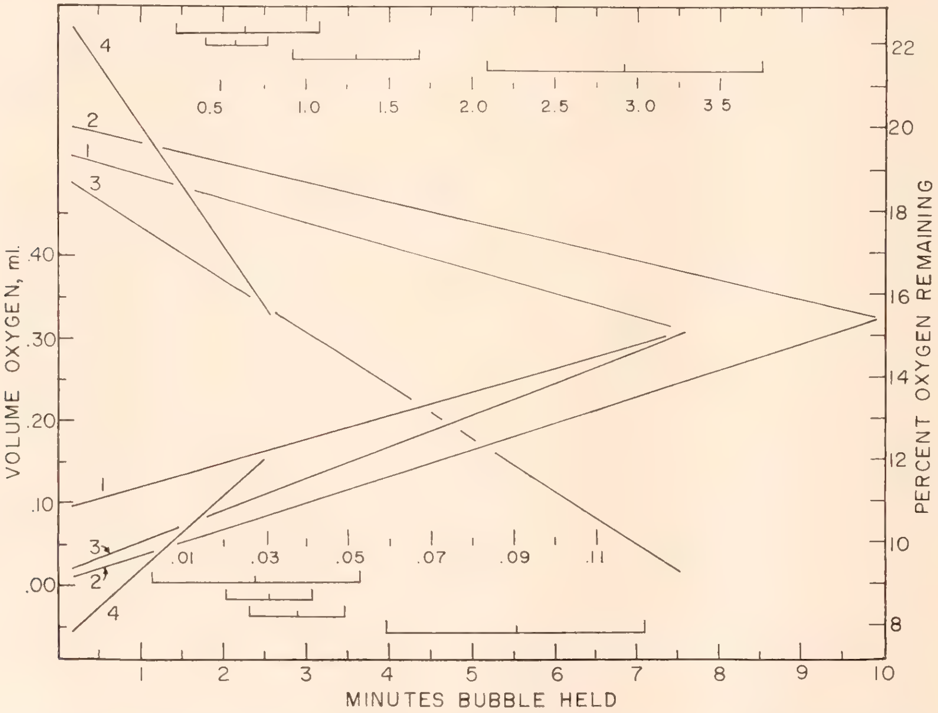


FIGURE 3. Least squares regressions of (right ordinate) per cent oxygen remaining in a volume of air in the funnel apex after the restrained mudsucker has released its bubble (upper four regression lines) and (left ordinate) the calculated volumes consumed during its bottom sojourn (lower four lines), on (abcissa) the time that each bubble was held. Numbers at the left extremes of the lines designate: 1, 165-mm. fish (7 observations while gulping in 6 ml. of air); 2, 140-mm. fish (8, in 6 ml.); 3, 117-mm. fish (12, in 3 ml.); 4, a second 117-mm. fish (9, in 3 ml.). The series of four measured horizontal lines at the top and bottom of the graph denote the regression coefficient "b" (centered short vertical line) and 95% confidence limits (horizontal extremes) for fish 1 (uppermost horizontal line in each series) through 4 (lowermost line). Non-overlap of any pairs of horizontal lines, therefore, indicates significant differences in b's (slopes of the lines). All regressions are significant at: 1, $P = 0.025$; 2–4, $P < 0.001$ levels.

depth by the fish. This volume, in turn, was erratically determined by the fish's success in securing an optimal volume in the initial gulp; *i.e.,* if too much air is retained (1.0 ml. or more), the fish is positively buoyant and cannot maintain its resting position on the bottom, and, conversely, if only a small amount of air is retained (less than 0.5 ml.), the fish lacks sufficient oxygen to allow it a reasonable sojourn on the bottom. Therefore, if too much was initially gulped, the positively buoyant fish would expel excess air during its descent or on first reaching the bottom. Occasionally the fish would overcompensate by expelling too much air and thereby shorten its bottom sojourn. When the funnel was removed, the duration of sojourn varied similarly; 0.5–10 minutes (average 3.8, usual range 2–5, 35 observations) compared with the above-mentioned durations under the funnel, 0.83–10 minutes (average 3.5, usual range 2.5–5.0, 36 observations). When a fish expelled part of a fresh bubble during hydrostatic adjustment, it was released from the mouth, operculum, or both. A respired bubble that was retained during the sojourn, however, was always released from the mouth.

For each trial, the regression (Fig. 3) of the percentage of oxygen in the funnel after expiration (Y) on duration of bubble retention (X) is highly significant ($P = 0.01–0.001$). Furthermore, the Y-intercepts ($a = 18.91–22.89$), which represent the theoretical percentage concentration of oxygn in the original inoculum of air, are reasonable values, even though that for one agitated 117-mm. specimen was significantly greater than the rest. To be sure, the obvious differences among regression coefficients ($-b = 1.297$ and 2.918 for the two smaller fish, 0.578 and 0.632 for the two larger) might partly be due to the fact that the smaller fish had less air to breathe than the larger; *i.e.,* all else being equal, the rate of percentage change in the small volume is necessarily greater (Fig. 3, horizontal lines at top). Therefore, the percentage decrease in aerial oxygen was converted to the presumed actual volumes consumed. Although less variable than those based on per cent change, the regressions of volumes on time held also indicate differences among regression coefficients (Fig. 3, horizontal lines at bottom), which of course are now positive ($+b = 0.038$ and 0.091 for the smaller fish, 0.026 and 0.031 for the larger). Both of the larger and one smaller fish consumed oxygen at about the same rate (as measured by the regression coefficients) throughout the experiment. The obvious hyperpnea in the second smaller fish, however, is substantiated by its behavior; it appeared agitated and had sojourns averaging only 1.73 minutes, compared with 3.77 minutes for the first and 5.11–5.27 for the two larger fish. Possible curvilinearity in some regressions is implied by the variation of Y-intercepts, which, of course, should theoretically equal zero.

Percentages and volumes of carbon dioxide increased in almost direct proportion with oxygen consumption (Fig. 4). The regression coefficient of carbon dioxide (Y) on oxygen (X), which essentially equals the respiratory quotient for the fish, however, was less (0.279) than would be expected if all gaseous exchange occurred in the pharyngobranchial epithelium. However, most of the carbon dioxide may have been excreted through the gill membrane (*cf.* Carter, 1957), some through the skin (*cf.* Krogh, 1904) and a significant amount dissolved in sea water while the bubble was being "mouthed" and expelled. (The theoretical average RQ, 0.83, for the metabolism of proteins, fats, and carbohydrates (*cf.* Baldwin, 1949) approximates the average of values, 0.76, obtained by Schöttle (1931) for the mudskipper *Periophthalmus* breathing in a closed system where exchange through all respiratory membranes was measured.)

In deoxygenated water under an atmosphere of pure nitrogen, the larger fish, after waiting 8–15 minutes, gulped and held the nitrogen bubble for an average of 5.40 minutes during 5 trials. But under a 1:1 mixture of nitrogen and carbon dioxide, they held for an average of only 34 seconds, then remained at the surface with their mouths above water. In an uncovered aquarium of oxygenated water aerated with nitrogen they were unaffected while the oxygen concentration was sufficient for aquatic respiration, but began gulping when it became critically low. When the aquarium was aerated with carbon dioxide, however, the fish gulped within 10 minutes and showed the dilation of buccopharyngeal vascularization that is always preparative for gulping.

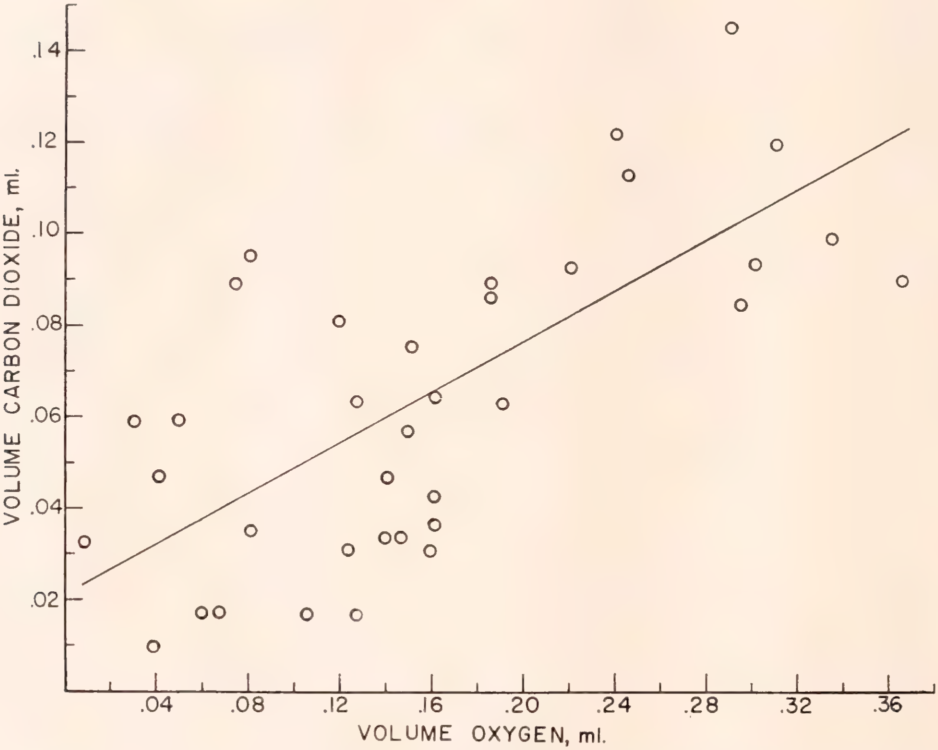


FIGURE 4. Regression of calculated volumes of carbon dioxide expired on those of aerial oxygen consumed per gulp for the four mudsuckers gulping under inverted funnels. The regression coefficient is 0.279 and the regression is significant at $P < 0.001$.

While gulping the same air for 30 minutes under the funnel, the larger fish increased the volume per gulp from 0.5–1.0 ml. to 1.0–2.0 ml. Near the end of this experiment, however, the fish gulped erratically, expelled bubbles quickly, and did not regularly either increase or decrease the volumes gulped. During this period, the oxygen in the funnel air decreased to 11% of the remaining volume and carbon dioxide increased to 3%.

An analysis of variance revealed no significant differences in respirometrically determined oxygen consumption between four half-grown fish breathing aerially in moist chambers and the same individuals gulping in chambers half-filled with sea water (Table I). Also, during the two experiments the lack of significant "within-fish" differences indicated no detectable change in rate of oxygen consumption with time. The significant differences among the four fish ($P = 0.01$) that were revealed in a two-way analysis eliminating replications (within-fish trials) were, perhaps, due to differences in excitability; whereas the two 8-gram fish consumed an average of 100 (range 80–136) and 91 (50–123) cc./kg./hr., respectively, the two 10-gram fish surprisingly consumed oxygen at a significantly faster rate: 113 (64–174) and 112 (63–172) cc./kg./hr. All fish averaged 104 (50–174) cc./kg./hr. for all trials. Our values approximated those obtained by Schöttle (1931), 94–210 (extrapolated to a body weight of 8–10 g. at 20° C.), for *Periophthalmus* and those of Wells (1935), 96–116, for aquatic breathing mudsuckers acclimated to 10.5–13.0° C. However, they were less than twice the average, 65 (interpolated at 20° C.), that Barlow (1961a) determined for emergent but moist fish breathing pure oxygen and Wells' range of values, 62–68, for aquatic breathers acclimated to 32.8–34.5° C. Whereas we as well as Schöttle accepted measurements taken during the first 2 hours of trials, Barlow rejected those of the first 12 hours, during which the rate of oxygen consumption generally decreases by half from an active to a standard rate. In evaluating the respiratory potential of the swimbladder, therefore, we assumed

TABLE I

Variation in oxygen consumption in Gillichthys mirabilis, *either emergent and moist or submergent and gulping, as expressed by an analysis of variance: upper, 3-way with the third-order interaction used as the "error" term and lower, 2-way with replications (trials) pooled and eliminated* (NS, not significant at the $P = 0.05$ level)*

Source of variability	Sum of squares	Degrees of freedom	Mean square	"f"	P
A. Among individual fish	10214.1	3	3404.7	4.2	0.025
B. Among replications (trials)	3488.2	9	387.6	<1	NS
C. Between treatments (emergent or submergent)	2063.8	1	2063.8	2.5	NS
Interactions A × B	17751.7	27	657.5	<1	NS
A × C	1635.0	3	545.0	<1	NS
B × C	10617.6	9	1179.7	1.4	NS
"Error" A × B × C	21949.8	27	813.0		
A. Individuals	10214.1	3	3404.7	4.55	0.01
C. Treatments	2063.8	1	2063.8	2.76	NS
Interaction: A × C	1635.0	3	545.0	<1	NS
Error	53807.2	72	747.3		

* The small number of samples (one/cell) disallowed a 3-way analysis with true error term.

that a half-grown mudsucker consumes oxygen at a standard rate of about 65 cc./kg./hr. and a large adult at about 45 cc./kg./hr.

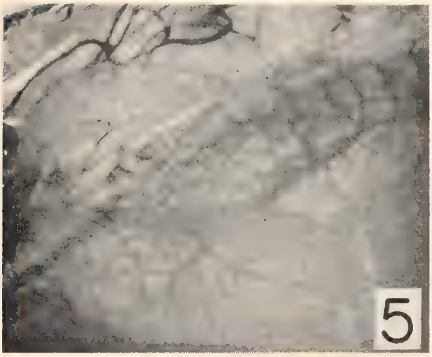
Structure of the buccopharyngeal organ

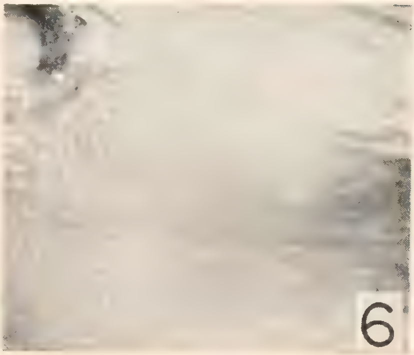
The large and loosely hinged jaws of *G. mirabilis* form a relatively huge gape, whose full lateral expansion measures almost half the body length and serves in a behavioral "combat pose" during courtship (Weisel, 1947). Its wide, heavily vascularized frenum could extend the aerial respiratory surface (Weisel), but our observations indicate that this membrane is not exposed to the air bubble. The marked depressibility of the floor of the mouth allows considerable expansion of the buccopharyngeal cavity. Unlike *Periophthalmus*, whose pharyngeal respiratory surface is extended by a fold of skin attaching the first gill arch (Schöttle, 1931), *Gillichthys* has four complete and normal gill arches. Like *Periophthalmus* and other air-breathing gobies, however, its gills are relatively short, comprise only about two-thirds the relative surface area of those of many obligatory aquatic species, and tend to clump during emergence.

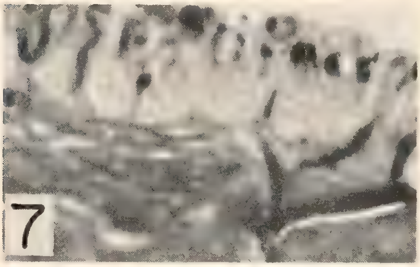
The capillary beds that permeate the buccopharyngeal organ are simply extensions of vessels whose homologues in obligatory aquatic breathers are not so proliferous. The epithelium is profusely vascularized, particularly a triangular area in the buccal roof, which contacts the top of the engulfed air bubble (Fig. 5). When injected with India ink or engorged with blood, this network is conspicuous compared with homologous vascularization in an obligatory aquatic breather such as *Fundulus* (Fig. 6). Even when uninjected, furthermore, the superficial vessels, which are enveloped in silvery-black pigment, are clearly visible, especially so in smaller specimens.

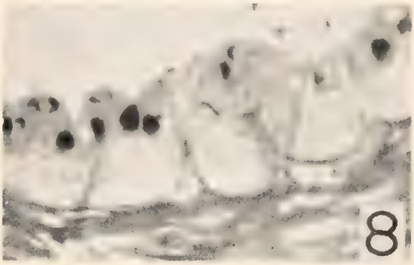
Afferent vessels supplying the respiratory epithelium include branches of the carotid, hyomandibular, hypobranchial, and segmental arteries. From the ventral aorta, blood courses the first efferent branchial artery to the hyomandibular artery, which, through its mandibular and lingual branches, supplies the rich capillary beds in the tongue and ventrolateral buccal epithelium. Also, the hyomandibular artery, together with hypobranchial branches of the second and third efferent branchial arteries, supply smaller networks in the pharyngeal epithelium. The vasculose superior buccal epithelium is fed by orbital, nasal, and smaller branches of the carotid arteries, which originate from the dorsal aorta at the first efferent branchial artery. Although in mudskippers the orbital and nasal arteries originate together from the orbitonasal branch of the internal carotid (Schöttle, 1931), in *G. mirabilis* no common source supplies these vessels, which originate separately from the internal and external carotids, respectively.

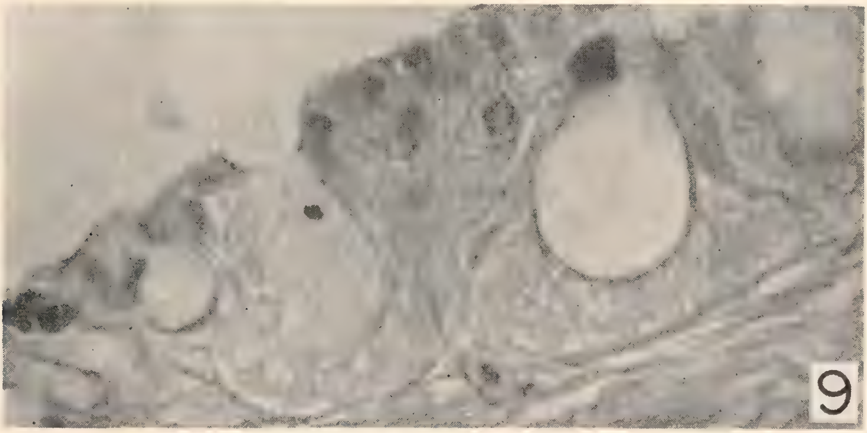
Efferent vessels draining the respiratory epithelium include the internal and external jugular veins, the orbital sinus, the opercular vein, and their tributaries. Lingual and other branches to the internal jugular vein drain the capillary beds in the tongue, inferior buccal epithelium, and hypobranchial region. The orbital sinus and external jugular trunk, which admit orbitonasal and nasomaxillary branches, respectively, receive blood from the superior buccal epithelium, while laterally, two large branches to the external jugular drain the periphery of this region. In the pharynx the large opercular vein and its tributaries drain into the anterior cardinal sinus, which also drains the orbital and external jugular sinuses.







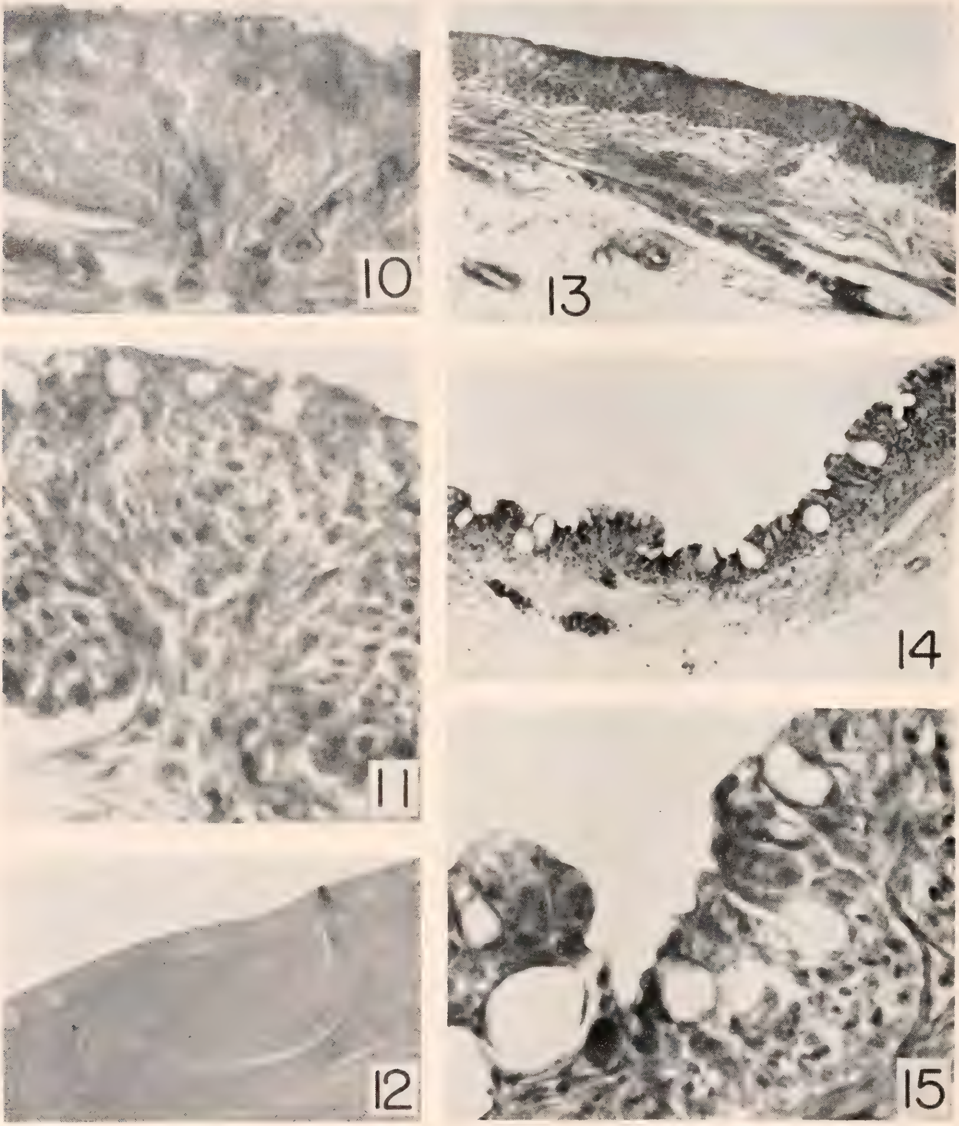




FIGURES 5, 6. Medio-lateral surfaces of the buccal roof from: 5, *Gillichthys mirabilis* and 6, *Fundulus parvipinnis* after both fishes were taken from deoxygenated water and injected intracardially with India ink. × 60.

FIGURES 7, 8. Cross-sections, from the postero-medial buccal roof, of respiratory epithelium (lighter upper tissue) and dermal corium (darker lower tissue), showing capillaries (dark vertical lines) extending from the corium to the epithelial surface. In: 7, from a mudsucker beginning to gulp in deoxygenated water, the capillaries are engorged with blood; 8, from a fish returned to aerated water, they are collapsed. × 430.

FIGURE 9. Cross-section of buccal-roof epithelium from a gulping mudsucker taken from deoxygenated water, showing enlarged vacuoles, one of which (to left of middle) is secreting mucus, interspersed cuboidal cells, and erythrocytes in engorged capillaries (*e.g.*, dark-staining cells near surface). × 1675.



Figures 10–15. Cross-sections of buccal-roof epithelium from a: 10, mudsucker taken from aerated water but with partly filled epithelial capillaries (× 1150); 11, fish from aerated water with collapsed capillaries and contracted vacuoles (× 1430); 12, killifish, *Fundulus parvipinnis,* from deoxygenated water, showing one taste bud (dark-tipped striated oval at right), but no epithelial capillaries and with small vacuoles under a smooth epithelial surface (× 1430); 13, mudsucker from aerated water with contracted vacuoles and a smooth epithelial surface (× 270); 14 (× 270), 15 (× 1150), fish from deoxygenated water with expanded vacuoles and corrugated epithelium. In all figures the strongly nucleated epithelium overlies the fibrous corium. The dermal and epithelial capillaries (tubular structures) contain erythrocytes (oval cells with darkly-staining nuclei). Several white vacuoles open to the epithelial surface.

Schöttle (1931) described three progressively more effective respiratory modifications of the buccopharyngeal epithelium in gobies: (1) the capillaries are relatively sparse and completely subepithelial in mostly aquatic *Gobius caninus* and *Periophthalmus koelreuteri*, (2) they are more numerous and extend from the subtending corium to the epithelium in amphibious *Boleophthalmus boddaerti*, and (3) they are completely epithelial in semi-terrestrial *P. schlosseri*. In the more competent air-breathers, extensive epithelial corrugation enhances this capillary profusion by greatly expanding the respiratory surface. The superior buccal epithelium of *G. mirabilis* most closely resembles the structurally intermediate type of *Boleophthalmus*, in that many capillaries extend from the dermal corium to the surface of the glandular epithelium, where they course among the vacuolated cells and often contact the surface membrane (Figs. 7, 8, 10). In contrast with the mudskipper, however, vascularization appears denser in the buccal (rather than pharyngeal) epithelium. Almost all capillaries in fish from deoxygenated water are expanded (Fig. 7), whereas those of fish in aerated water appear mostly collapsed (Fig. 8).

A comparison of histological sections from the buccal roof of *G. mirabilis* with others from obligatory aquatic *Fundulus parvipinnis* revealed manifold differences, especially among specimens from deoxygenated water. Within 10 intervals of 6 mm. each, *Gillichthys* averaged 65 epithelial capillaries, many of which approached the surface, whereas *Fundulus* had few or none contacting the epithelium and those that did were often associated with taste buds. The epithelium of both species is composed of cuboidal cells and interspersed mucus-secreting goblet cells (Figs. 9–11). Specimens of both from aerated water have smooth epitheliums containing relatively small vacuoles (Figs. 11–13). But among others from deoxygenated water, only specimens of *Gillichthys* showed a noticeable epithelial transformation; vacuoles were enlarged to 5–10 times normal size (Figs. 9, 14, 15), and the numerous epithelial capillaries were engorged with blood. In this condition it resembled the thickened corrugated epithelium of *P. schlosseri*, which is similarly permeated by branched, alveolar-like cavities and engorged capillaries (*cf.* Schöttle, 1931; Harms, 1935). When fully expanded, the vacuoles open to the epithelial surface and exude over it a copious mucus (Fig. 9), which may provide the fluid medium of respiratory exchange.

When *Gillichthys* enters either deoxygenated water, water aerated with carbon dioxide, or air, its buccopharyngeal capillaries, as they become engorged with blood, dilate and redden. Within 3–5 minutes superficial capillaries impart a rosy hue to the tongue and buccal roof; conversion of the larger vessels is completed 10–20 minutes later. Although this response is relatively rapid, its reversal in aerated water requires several hours or occasionally even days, during which the red-mouthed, transient fish are easily distinguishable from the white-mouthed residents and often gulp sporadically. No such vascular transformation accommodates the buccopharyngeal epithelium of *Fundulus*.

Composition of gas in the swimbladder

In the mudsucker's physoclistic swimbladder, the glandular part (red gland), which comprises a round gas gland and long unipolar rete mirabile (series of

TABLE II

Proportions of swimbladder oxygen in gulping and aquatically breathing mudsuckers among various habitats (average precedes the range, in parentheses, and number of individuals sampled)

Habitat	Per cent oxygen in swimbladders of fish:	
	Gulping in low oxygen, respiratory capillaries engorged with blood	Respiring aquatically in normal oxygen, capillaries not engorged
Aquarium, 1 month	17.9(15.5–20.8)6	26.2(18.0–34.4)10
Large concrete tank, 1 month	22.5(22.0–23.5)3	32.7(30.5–34.0)3
Campus lagoon, immediately after capture	16.0(14.0–26.0)6	25.7(24.3–28.4)6
Grand average	18.1	27.1

minute, juxtaposed capillaries where gases are concentrated) is well developed and, therefore, should have a relatively good capacity for oxygen secretion.

After the two groups of four fish each had been transferred from an aerated aquarium into either deoxygenated or continuously aerated sea water, their swimbladder gas differed markedly in oxygen content. Swimbladders of fish in aerated water contained 40.5–43.0% 15 hours after transfer, whereas those of fish in deoxygenated water contained significantly less: 19.0–23.5% in two fish after 27–29 minutes, 27.2% in one after 80 minutes, and 21.0% in the last after 15 hours. The group in deoxygenated water remained quietly submerged without noticeable opercular movement for 8–33 (average 15.3) minutes before gulping. In a third

TABLE III

Proportions of newly secreted oxygen and carbon dioxide in refilled mudsucker swimbladders at selected intervals after the forced removal of the original gas

	Time after removal, hours			
	6 (Residual gas?)	12 (Refilled normal volume)	24	48
Oxygen, %	33.6	85.4	85.5	41.5
	31.0	85.4	84.3	39.3
	31.9	81.4	73.3	56.5
	39.5	84.2	81.4	73.0
	23.0	78.3	84.4	57.5
Average	31.8	82.9	81.8	55.0
Carbon dioxide, %	2.6	13.5	12.0	4.2
	4.5	9.3	10.0	6.0
	3.7	13.0	3.2	4.0
	3.5	6.2	2.6	5.0
	5.0	9.5	3.2	5.5
Average	3.9	10.3	6.2	4.9

group placed in deoxygenated water, swimbladder oxygen fell from an average of 28.5% to 14.0% in 17 minutes, their average time to gulping. In deoxygenated water, obligatory aquatic-breathing killifish, *Fundulus parvipinnis*, and topsmelt, *Atherinops affinis*, died in less than 15 minutes, during which no proportional change in their swimbladder gas content was detected.

Swimbladder gas in 19 fish gulping in poorly oxygenated water among different environments averaged only 18.1% oxygen, compared with 27.1% in fish (excluding those with recently filled swimbladders) from normally oxygenated water (more than 5.0 mg./l.) in similar environments (Table II). The percentage volume of carbon dioxide averaged 2.7% in the fish from poorly oxygenated water, 2.3% in those from normal water. After 20 minutes in deoxygenated water under a nitrogenous atmosphere, one fish, after remaining submerged for 15 minutes before gulping, had 6.0% oxygen in its swimbladder; another, which gulped after 45 minutes, but 1.5%.

Six hours after they had been deflated, swimbladders in 5 fish contained only about 10–14% of the filled volume of gas. Because the gas averaged only 31.8% oxygen and 3.9% carbon dioxide, furthermore, it was assumed that most of it was residue from incompletely deflated organs. In 12 hours the swimbladders had completely refilled with gas averaging 82.9% oxygen and 10.3% carbon dioxide (Table III). Subsequently, however, the percentages decreased proportionately to 48 hours, when oxygen averaged 55.0% and carbon dioxide 4.9%, as compared with "normal" averages of 26.2% and 2.3%, respectively (Tables II, III).

The volumes of 5 swimbladders measured by attempted complete gas removal into a calibrated syringe constituted 3.0–3.6% of the body volumes.

Discussion

Carter (1957) proposed three criteria that ideally should be satisfied in determining the respiratory capacity of suspected internal air-breathing organs in fishes: (1) the organ's epithelium should be richly supplied with blood which is better oxygenated after leaving the organ, (2) its cavity should be regularly ventilated, and (3) its expelled air should contain proportionately less oxygen and more carbon dioxide than atmospheric air. Also, he acknowledged the importance of biological and behavioral evidence, such as an established pattern of presumed air-breathing movements and the ability to survive in deoxygenated water if allowed access to surface air (or inability if denied such access). Of these, we have investigated all but the criterion of blood gas concentrations, which will be the subject of later studies.

Although all fishes tend to gulp or gasp at the surface in deoxygenated water (*e.g.*, Fry, 1957; Jones, 1964), only the species specifically adapted for aerial respiration can continuously survive by such actions. When dissolved oxygen falls below 2.0 mg./l., for example, an adult mudsucker usually gulps air at the water surface (often after a preliminary sequence of abortive "intention movements"), holding the bubble in its large buccopharyngeal cavity and retiring to the bottom for varying periods until the bubble is expelled. If denied surface access it suffocates if the surrounding concentration of dissolved oxygen is less than 1.6 mg./l. Among other fishes, similar responses are achieved by the anabantoids, such as the paradisefish *Macropodus opercularis* of the swamps and rice paddies of southeastern

Asia, whose air-breathing organ comprises complex, heavily vascularized lamellae set in a spacious suprabranchial cavity (*e.g.*, Bader, 1937; Forselius, 1957).

Thompson (1925) concluded that water containing less than 2.0 p.p.m. (mg./l.) of oxygen will kill many kinds of obligatory aquatic breathers and, according to Ellis (1937), "ample and favorable" natural waters should contain more than 5.0 mg./l. of oxygen to be generally suitable for most fishes. Jones pointed out that previous work has shown the tolerance of low oxygen tensions to be a characteristic that is best developed in relatively sluggish fishes and is strongly influenced by the ambient temperature, although even for these types optimal concentrations are much greater than the lowest tensions survived. The low tensions that induce aerial respiration in *G. mirabilis* approximate critical tensions that cause discomfort, suspension of normal activities, and often death in many non-air-breathers. Also, in contrast with most fishes, which are oxykinetic (made restless by decreasing oxygen) and are thereby stimulated to randomly seek better oxygenated water (Hoglund, 1961; Jones, 1964), the adult mudsucker becomes quite still in critically low oxygen tensions and ceases all opercular movements for several minutes before gulping at the surface. This characteristic period of adjustment allows the functional-morphological changes necessary for aerial respiration to proceed to completion.

Air-breathing fishes must somehow compensate for their added buoyancy derived from the gas in their respiratory organ. The gulping mudsucker may accommodate this by attaching itself to the bottom with its fused pelvic fins. If it takes a larger than optimal gulp, it often somewhat erratically expels excess air through its gill cover, although the respired bubbles are always released through the mouth. This means of buoyancy adjustment may serve a dual purpose, in that Carter (1957) noted that most fishes occasionally gulp air and expel it through the gill opening simply to flush the gills of clogging substances. The relative volume of the mudsucker swimbladder is significantly less (3.6–4% of its body volume) than that (about 5%) which, according to Jones and Marshall (1953), is necessary for maintenance of hydrostatic equilibrium in most marine teleosts. But the engulfed bubble usually occupies about 1% of the body volume, so that between gulps, fish are almost neutrally buoyant and normally must attach to the bottom. After the bubble has been expelled, however, these benthic fishes tend to sink.

Next, we have shown that a continuous decrease in oxygen and concomitant increase in carbon dioxide in the engulfed bubble is almost directly proportional to the time that it is held. Schöttle (1931) analyzed similar changes in the concentrations of these gases held continuously in the buccopharynx of the mudskippers *Periophthalmus* and *Boleophthalmus* while they were breathing in "free air." Their active rate of oxygen consumption approximated that determined in the present study for half-grown fish, which consume oxygen at about the same rate whether they are breathing aerially while emergent or gulping, and others presented by Wells (1935) for fish breathing aquatically. The standard rate of oxygen consumption determined by Barlow (1961a) for environmentally adjusted fish breathing pure oxygen in moist chambers is about half the active rate. The volumetrically determined respiratory quotient for mudsuckers, about 0.3, is significantly less than the expected value, which theoretically should be only slightly less than unity. However, this value is reasonable considering the potential loss of carbon dioxide to the surrounding sea water both directly and by diffusion through the gills, or perhaps, skin. The volumetric relationship of these gases during gulping, therefore, strongly indicates the occurrence of considerable respiratory exchange in the buccopharyngeal cavity.

The usual stimulus for gulping is most likely the presence of critically low dissolved oxygen tensions, though mudsuckers do respond to abnormally high concentrations of carbon dioxide. That ordinary environmental levels of carbon dioxide can generally stimulate breathing in most fishes, however, is uncertain (reviewed by Fry, 1957; Jones, 1964). Carter (1957) summarized earlier studies that indicated internal air-breathing organs in fishes to contain as much as 3% carbon dioxide; this would prevent full oxygenation of ordinary hemoglobin. After an especially long sojourn on the bottom, air-breathing mudsuckers have up to 4.0% carbon dioxide in their buccopharynx. Willmer (1934) and Fish (1956) showed that some air-breathing fishes are less sensitive to carbon dioxide than obligatory aquatic breathers and Carter suggested that most air-breathers may have evolved a hemoglobin relatively insensitive to this gas. Although *G. mirabilis* gulps normally under pure nitrogen, it gasps erratically under an atmosphere of equal parts nitrogen and carbon dioxide. In oxygenated water it is initially unaffected by bubbled nitrogen and gulps in a few hours, only after the oxygen concentration has become critically low. When in sea water aerated with carbon dioxide, however, the fish gulps after a normal period of adjustment and soon develops the "red mouth" typical of air-breathing individuals. When it continues gulping in a small unrenewed volume of air, gulps progressively increase in volume and erratic gasping occurs. In this confinement carbon dioxide may rise to 3% or 4% as the oxygen content decreases. Therefore, we suggest that dissolved carbon dioxide in sufficient quantities may reinforce the effects of depleted oxygen in initiating gulping and that relatively high concentrations in air cause hyperpnea.

The buccopharyngeal epithelium of mudsuckers is obviously specialized for respiratory blood-vascular transport, in that its superficial proliferation of capillaries is greatest between the tongue and buccal roof, where the air bubble is held. As in other air-breathing gobies (and air-breathing fishes in general), this vascularization is simply a modification of homologous systems in non-air-breathing relatives and presents no innovation in general systemic circulation (*cf.* Schöttle, 1931). Mott (1957, p. 95) assumed that because this type of ". . . accessory respiratory circuit is . . . in parallel with systemic circuits," the system is relatively inefficient, in that a parcel of oxygenated blood may never make a complete circuit. Nevertheless, Carter (1957) pointed out that because air contains proportionately much more oxygen than does water, any greater efficiency is obviated in air-breathers.

The extensive morphological preparation for buccopharyngeal respiration may occur during the mudsucker's quiescent period of adjustment before gulping. Not only is the potential blood supply increased during this period, but also, related histological changes are initiated, which corrugate, lubricate, and pit the epithelium. Similar but long-term changes, which can be accelerated by chronic injections of thyroxin, convert the buccopharyngeal epithelium in *Periophthalmus* (Harms, 1935). Therefore, to survive this critical period the mudsucker must have an auxiliary oxygen supply.

The apparent absence in most instances of all opercular movement probably eliminates the possible use of gills during this period, when oxygen tensions are already much less than optimal for aquatic respiration. Cutaneous respiration, as observed in several gobies (*e.g.*, in *Neogobius* by Shulman, 1956), is improbable as the major source, also because of insufficient dissolved oxygen. Noting that some Salton Sea mudsuckers with blood-engorged fins move into shoal, better-oxygenated waters at night, however, Walker *et al.* (1961) suggested that a combination of behavioral and physiological (*i.e.*, cutaneous respiration, especially through the fin membranes) mechanisms might help fish endure low nocturnal oxygen tensions.

It is possible that the mudsucker assumes a naturally induced state of torpor or that it metabolizes anaerobically during the adjustment interval. The crucian carp *Cyprinus carpio* can tolerate anoxia for a considerable period (Blažka, 1958). Blažka found that when it was transferred into deoxygenated water it would rest quietly on the bottom of the tank and continue its opercular movements, albeit not so vigorously as before. At 15–20° C., fish endured anoxia for 2–33 hours, depending on their degree of acclimation to low oxygen tensions; but at 5° C., fish remained quietly in this state for two months "without danger." The species is biochemically adapted to survive anoxia, in that the end-products of anaerobic metabolic metabolism are mostly fats, not noxious lower fatty acids, etc. that accumulate in species (*e.g.*, the trout) metabolically ill-adapted to survive anoxia. Nevertheless, he concluded that at higher temperatures anaerobic metabolism yields insufficient energy, even to sustain the carp for long periods. Beadle (1961) further reviewed the mechanisms and consequences of anaerobic metabolism in animals. Animals such as the carp that have evolved mechanisms of anaerobiosis, however, usually lack elaborate means of aerial respiration such as the buccopharyngeal organ of the mudsucker. Furthermore, unlike the carp which must survive the winter in stagnant, often frozen-over ponds, the mudsucker needs endure anoxia only minutes. Although it, like the carp, rests quietly on the bottom when subjected to anoxia, it is certainly not torpid and can dart away at the slightest provocation. For the mudsucker, a similar and therefore more likely mechanism for surviving the quiescent adjustment interval presumes the tapping of an already available though limited oxygen supply, which, before the initial gulp, could only be obtained from the swimbladder. As blood and tissue oxygen is depleted, swimbladder oxygen would automatically enter by simple diffusion through the permeable membrane of the red gland or oval, thereby rendering a more complex biochemical system unnecessary.

The gas content of the physoclistic swimbladder implies its possible function as a respiratory organ, at least in an auxiliary capacity (*e.g.*, Popta, 1910; Fänge, 1953). Berg and Steen (1965) stated (p. 470) that "The oxygen content of the swimbladder must . . . be taken into consideration when the reactions of fishes to situations of asphyxia are studied." When physoclists are held in poorly oxygenated water, the proportion of oxygen in the bladder decreases while carbon dioxide increases (reviewed in Jones and Marshall, 1953; Jones, 1957). Fänge (1953) further demonstrated that this "deflatory response" is caused by either an environmental oxygen deficiency or an excess of carbon dioxide. Other investigators, however, have concluded that because the extra oxygen removed from the bladder would meet the fish's respiratory requirements for only a few (5–15) minutes, its respiratory function cannot be important relative to the total amount required for continued metabolic needs (reviewed in Jones and Marshall, 1953; Jones, 1957). Nevertheless, Jones (1957) suggested that shallow-living physoclists may draw on swimbladder oxygen in times of stress, and the general consensus predicts that they could depend on swimbladder oxygen for short respiratory emergencies caused by critically low oxygen and/or abnormally high carbon dioxide tensions.

As in other fishes, the swimbladder oxygen content of *G. mirabilis* decreases in deoxygenated water, usually by more than half preparatory to gulping and by as much as 95% under an atmosphere of pure nitrogen. As in others (*cf.* Kuhn *et al.*, 1963), newly secreted gas is rich in oxygen and contains appreciable amounts of carbon dioxide, although both gases decrease proportionately to about 25–30% and 2–3% of the swimbladder volume, respectively, when the fish are kept in aerated water. Now, assuming that 60% of this oxygen is normally used as an emergency supply during the pre-gulping quiescent period of standard metabolism, a 30-g. adult could respire for 13 minutes during this period, as compared with 17 minutes, the average for all mudsuckers examined. Therefore, their small but well-developed swimbladder, which probably cannot maintain neutral buoyancy in these bottom-dwelling fish, might supply a large part of the required oxygen during the fish's period of adjustment to aerial respiration.

Preliminary observations of closely related *Gillichthys seta* indicate that it gulps in much the same way as *G. mirabilis.* Only half the size of *G. mirabilis*, the adults of *G. seta* resemble halfgrown of *G. mirabilis*, in that they apparently do not cease all opercular movements preparatory to and during gulping, and more readily substitute surface-breathing for bottom sojourns. The swimbladder of *G. seta* resembles that of *G. mirabilis* and contains respiratory gases in similar proportions. In the Gulf of California, another related goby, *Quietula guaymasiae* (Jenkens and Evermann) gulps surface air more simply. Its opercular movements continue at a rapid beat even in deoxygenated water. While holding the bubble in its buccopharynx, it remains at the surface for extended periods with its snout barely projecting. Its buccopharyngeal vascularization is less profuse than that of *Gillichthys* and because it lacks a swimbladder it has no auxiliary oxygen store.

Barlow (1961a, 1961b) suggested that adult *Gillichthys seta* resembles half-grown *G. mirabilis* in several morphological and physiological characteristics. Our observations of the gulping response tend to support this. The response of *Quietula guaymasiae* is even more generalized and resembles that in the young of *G. mirabilis.* As implied by Schöttle (1931), gobies may be preadapted to aerial respiration; many have evolved generalized to highly specialized mechanisms in response to individual trends in natural selection and exploitation of "extreme" habitats.

We thank Dr. Vernon I. Cheadle, Chancellor of U.C.S.B., for his continued support of our studies of local estuarine environments; Messrs. Andrew Kier, David Valentine and other students in the Department of Biological Sciences for technical assistance; and Drs. George W. Barlow, University of Illinois, and James F. Case, U.C.S.B., for critically reviewing the manuscript and for technical advice.

SUMMARY

1. Like several other estuarine gobies, *G. mirabilis,* which ranges from central California through the Gulf of California, can breathe aerially by means of its heavily vascularized buccopharynx.

2. When environmental dissolved oxygen falls below 2.0 mg./l., the fish ceases all opercular movements during a preliminary period of adjustment, then gulps mouthfuls of air at the surface, usually retires to the bottom for a brief sojourn, and expels the respired air before the next gulp.

3. While gulping in the confinement of an inverted funnel, the fish consume oxygen and excrete carbon dioxide in almost direct proportion with the time that the gulped air bubble is held. The relatively low respiratory quotient (RQ) suggests that considerable carbon dioxide is excreted by the gills and, perhaps, skin, even when the fish is gulping. Respirometric measurements indicate rates of oxygen consumption to be similar for all active air-breathing mudsuckers, whether submergent and gulping or emergent and moist.

4. Its spacious buccopharynx is heavily vascularized, especially between the tongue and buccal roof, where the bubble is usually centered. The proliferation of capillaries in the buccopharyngeal epithelium simply represents an evolutionary expansion of homologous systems present in non-air-breathing relatives, rather than an evolutionary innovation. When fish are placed in water low in oxygen, histological changes are initiated which dilate the epithelial capillaries, engorging them with blood, and which corrugate, lubricate, and pit the epithelium, thereby creating a lung-like surface. Within a few minutes after transfer to deoxygenated water, the mouth is reddened by engorged vessels, although full functional adaptation is attained 10–20 minutes later when the fish begins gulping. An obligatory aquatic breather, *Fundulus parvipinnis,* exhibits no such changes.

5. Because during its pregulping period of adjustment the adult mudsucker ceases all opercular movements, an auxiliary oxygen store most likely supplies oxygen for continued aerobic metabolism. The obvious source in the swimbladder could sustain the fish at a standard rate for all or most of this period and, indeed, the percentage of oxygen here decreases by considerably more than half during this time. After being forcefully emptied, the swimbladder is refilled in 12 hours with gas of high oxygen and carbon dioxide concentrations, which decrease to normal after 48 hours. The relatively small swimbladder cannot create neutral buoyancy for the bottom-dwelling mudsucker unless it is augmented by the volume of an unusually large gulp of air.

6. Therefore, the mudsucker conforms to all necessary and sufficient criteria thus far investigated for air-breathing in fishes. Although relatively sluggish, the aquatic-breathing fish tolerates about the same low oxygen tensions as most other species.

7. The Gobiidae include several air-breathing species, among which the mudsucker is intermediate in specialization for aerial respiration between closely related but relatively unspecialized *Quietula guaymasiae* of the Gulf of California and semi-terrestrial *Periophthalmus schlosseri* of southeastern Asia. Its only congener, *G. seta,* resembles it in gulping habit, but exhibits several juvenile characteristics, such as its continuation of opercular movements while air-breathing.

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CAPRELLA GRAHAMI, A NEW SPECIES OF CAPRELLID (CRUSTACEA: AMPHIPODA) COMMENSAL WITH STARFISHES

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A new species of caprellid amphipod that lives commensally with starfishes of the family Asteriidae was found in coastal waters of southern New England. The first specimens were detected in collections of marine invertebrates dredged in Vineyard Sound, off Falmouth, Massachusetts, by personnel of the Marine Biological Laboratory. These collections were made in March, 1965; subsequent collections in April and June, 1965, demonstrated that the species occurs in Great Harbor at Woods Hole, the passages between Vineyard Sound and Buzzards Bay, and the Cape Cod Canal. Specimens from Long Island Sound were collected by Clyde L. MacKenzie, Bureau of Commercial Fisheries Biological Laboratory, Milford, Connecticut. The restricted association of these caprellids with starfishes prompted a close examination of this amphipod species.

This appears to be the only known commensal relationship between a caprellid and starfishes in the Western Atlantic region.

We thank John J. Valois and David H. Graham, Marine Biological Laboratory, Woods Hole, for their assistance in obtaining starfish and caprellids used in this study; Thomas E. Bowman and John C. McCain for suggestions for improving the manuscript and bringing to our attention the specimens collected by Clyde L. MacKenzie, Jr., in Long Island Sound; and Frank A. Bailey for inking the drawings.

Caprella grahami[1] new species

Figures 1–5

Diagnosis

Moderately large *Caprella* with head rounded anteriorly; in male, distal segments of peduncle of antenna 1 densely covered with minute setae; body segments without setae, spines, or tubercles; maxillipeds, gnathopods, and pereopods with stout dactyls; propodus of posterior three pairs of pereopods without spines and with only a few setae, palm margin slightly convex to slightly concave.

Descriptions and illustrations of the genus *Caprella* are given by Mayer (1903), Kunkel (1918), and Steinberg and Dougherty (1957).

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[1] Named in honor of Dr. Herbert W. Graham, Laboratory Director, Bureau of Commercial Fisheries Biological Laboratory, Woods Hole, Massachusetts.

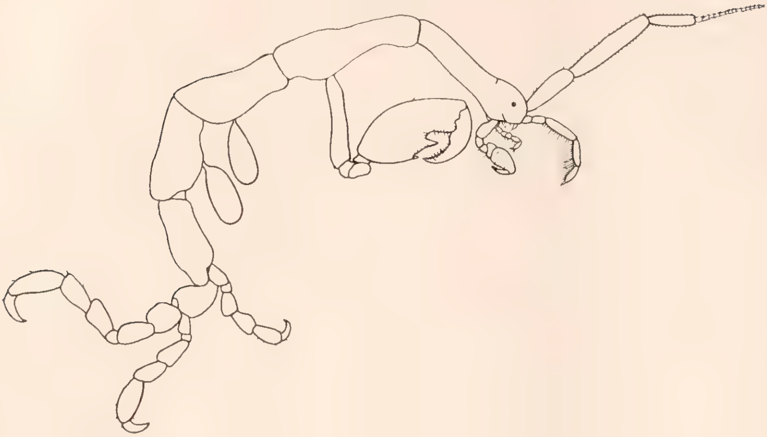

Figure 1. *Caprella grahami* new species, lateral view of adult male.

Description of male (Figs. 1–3)

Head elongate, rounded anteriorly, section posterior to groove one to two times as long as anterior section, posterior section shortest in small specimens. Pereods 2–4 slightly inflated ventrally and laterally. Pereods 2–7 smooth, without setae, spines, or tubercles. Cuticle of pereods and appendages, or portions thereof, in some specimens sculptured with small disc-like markings. Pereod 2 longest, equal in length to head; pereods 3 and 4 equal in length, about ⅔ as long as pereod 2; pereods 5–7 progressively shorter.

Antenna 1 large, nearly ½ body length; peduncle rather stout, distal segments densely covered with small setae; distal peduncular segment with small spine-like projection on anterodorsal margin; distal peduncular segment slightly shorter than basal segment; middle segment twice as long as distal segment; flagellum 9 to

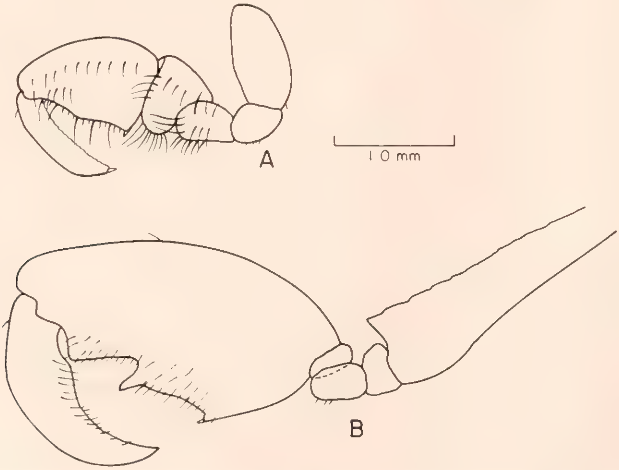

Figure 2. *Caprella grahami* new species, adult male (A) gnathopod 1 and (B) gnathopod 2.

14 segmented, flagellum approximately $\frac{1}{3}$ total antenna length. Antenna 2, about $\frac{1}{2}$ the length of antenna 1, extending to middle or distal end of second peduncular segment of antenna 1; peduncular segment 1 with lobe-like projection extending

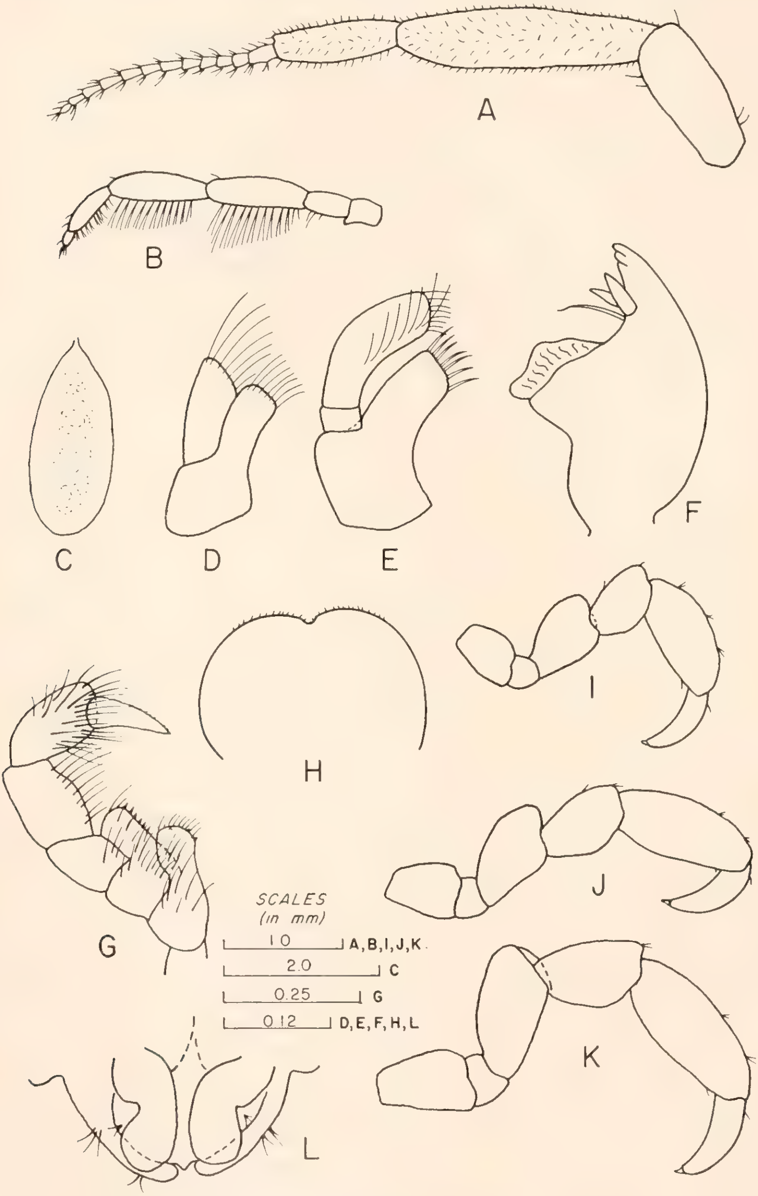

FIGURE 3. *Caprella grahami* new species, adult male (A) antenna 1, (B) antenna 2, (C) gill, (D) maxilla 2, (E) maxilla 1, (F) mandible, (G) maxilliped, (H) upper lip, (I) pereopod of 5th pereod, (J) pereopod of 6th pereod, (K) pereopod of 7th pereod, and (L) pleon.

anteriorly from anteroventral margin; peduncular segments 3–5 with row of long paired setae along ventral margin, longest on segment 3, moderately long on 4, shortest on 5; flagellum consists of a single segment.

Mouthparts typical of the genus. Maxillipeds with stout dactyls; maxilla 1 with longitudinal row of setae on distal segment of palp.

Gnathopod 1 small, subchelate; dactyl relatively large and broad, occluded margin minutely serrate, spine inset at apex; propodus broad (width equals $\frac{2}{3}$ length), setae on inner lateral face, palm margin nearly straight, bearing long and short setae, small spine at proximal end. Gnathopod 2 very large, subchelate; attachment to body located on posterior half of pereod 2; dactyl long and broad, occluded margin entire and setose, outer (anterior) margin on some specimens with tiny knobs. Propodus long and broad (width equals $\frac{1}{2}$ length), configuration of palm margin includes deep sinus midway along palm that separates broad, angular lobe distally from large tooth-like projection ("poison tooth") proximally; at proximal end of palm margin a small spine arises from moderate-sized lobe; broad but shallow excavation with straight margin separates "poison tooth" from proximal spine; small spine on inner face of propodus just above and slightly distal to large proximal spine. Carpus very small, merus and ischium small. Basis medium to long ($\frac{1}{2}$ to $\frac{3}{4}$ propodus length) and narrow (about two to four times maximum width), anterior margin finely irregularly sinuate, distal anterior corner sharply angular.

Gills elliptical to obovate; size moderate, length about equal to or greater than $\frac{1}{2}$ their pereod lengths.

Pereopods on pereods 5, 6, and 7 consist of 6 segments; all segments medium to moderately stout; dactyls broad; propodus large, length about $2\frac{1}{2}$ times maximum width; propodus without spines, and setae exceedingly sparse, palm margin usually slightly concave but sometimes straight or slightly convex.

Pleon minute. Uropod 1 comparatively wide, without setae, apex broadly rounded and entire. Uropod 2 narrow, sparsely setose, apex rounded and entire.

Description of female (Figs. 4 and 5)

Females are morphologically similar to males in many features; only the principal differences are mentioned here. Head similar to that of males but much shorter, section posterior to groove approximately equal in length to anterior section. Attachment of gnathopod 2 located on anterior half of pereod 2 (rather than on posterior half as in males). Peduncle of antenna not densely covered with setae. Flagellum consists of 8 to 10 segments. Antenna 2 proportionately longer than in

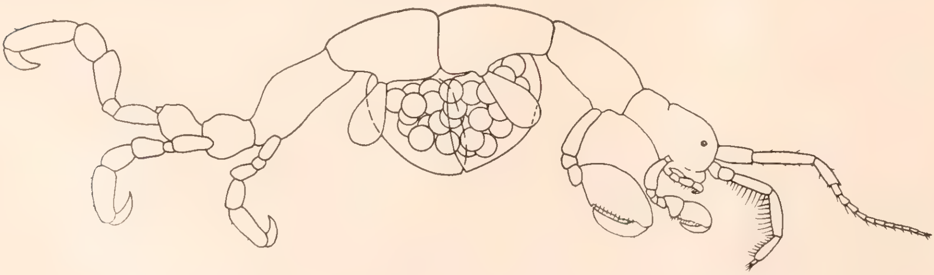

FIGURE 4. *Caprella grahami* new species, lateral view of adult female.

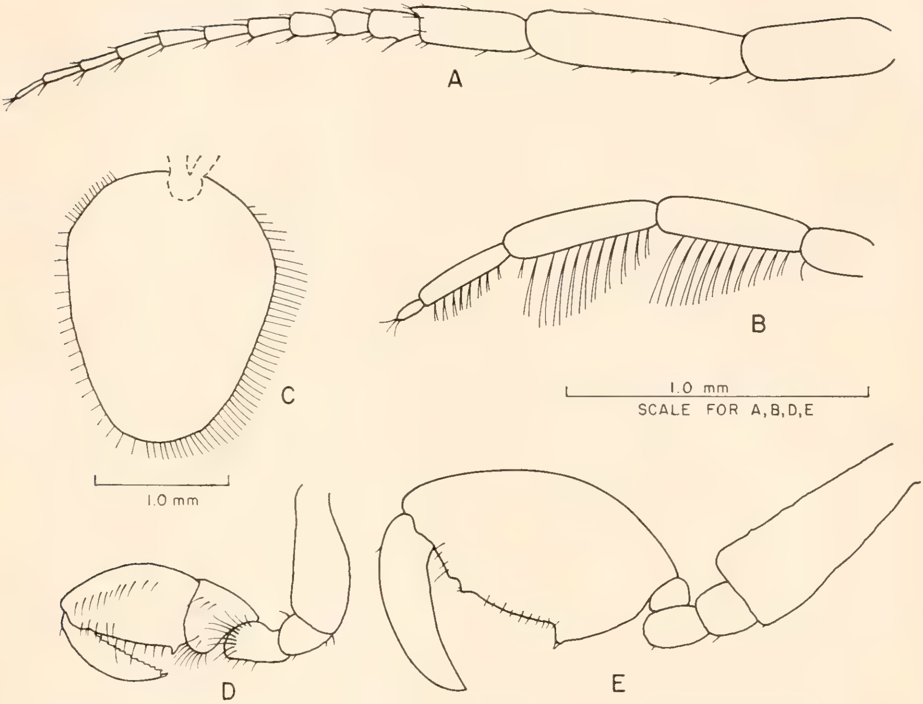

FIGURE 5. *Caprella grahami* new species, adult female (A) antenna 1, (B) antenna 2, (C) oostegite of pereod 3, (D) gnathopod 1, and (E) gnathopod 2.

male, nearly $\frac{3}{4}$ the length of antenna 1 or $\frac{1}{3}$ the total body length. Gnathopods proportionately smaller, particularly gnathopod 2; propodus palm margin of gnathopod 2 convex (without sinus or excavation), with small relatively broad tooth in middle of palm margin, and spine at proximal end. Pereod 2 relatively shorter; pereods 3 and 4 expanded laterally. Oostegites on pereods 3 and 4 large, slightly overlap one another medially and laterally; entire margin, except mid-dorsal section, fringed with setae; oostegite of pereod 3 with marginal setae comparatively long, particularly on posterior margin, and a brush-like group of setae on anterodorsal margin; oostegite of pereod 4 with minute marginal setae.

Shape of ova varied from slightly oblong to spherical. Ova size ranged from 0.38 to 0.46 mm., based on the measurement (maximum length) of 10 ova each from three females. Numbers of ova in three specimens were: 42, 44, and 47.

Juvenile specimens

Proportional sizes of appendages and body segments of juvenile specimens (2 to $3\frac{1}{2}$ mm., body length) differ substantially from those of adults. Antenna 1 incompletely developed, only slightly larger than antenna 2. Flagellum of antenna 1 composed of three segments; flagellum of antenna 2 one-segmented. Head short, posterior section about half as long as anterior section. Pereods relatively very stout, depth of pereod 2 equals its length. Gnathopods proportionately large but lack shape characteristic of adults. Pereopods also proportionately large, reveal some of the most distinctive features that characterize this species. Consequently, pereopods are one of the key components to be examined in identifying juvenile specimens of *C. grahami.*

Size

Body lengths of preserved specimens of *C. grahami* range from 2 to 18 mm. (Table I). Males are larger than females; maximum lengths observed for each are 18 and 8 mm., respectively. Minimum body length for both sexes is $3\frac{1}{2}$ mm. Males generally average between 8 and 12 mm., females average 5 to 7 mm., and juveniles range from 2 to $3\frac{1}{2}$ mm. The development of adult characteristics becomes evident in both sexes at body lengths of $3\frac{1}{2}$ to 4 mm.

TABLE I

Range in body lengths of C. grahami
(Length measured from the anterior margin of head to posterior tip of pleon; number of specimens measured is shown in parentheses)

Date of collection (1965)	Body length (mm.)		
	Males	Females	Juveniles
March 6	$5\frac{1}{2}$–11 (8)	4–$6\frac{1}{2}$ (4)	—
March 25	—	–8 (1)	—
March 29	4–$8\frac{1}{2}$ (6)	$3\frac{1}{2}$–7 (12)	$2\frac{1}{2}$–$3\frac{1}{2}$ (7)
March 30	$5\frac{1}{2}$–18 (17)	5–$7\frac{1}{2}$ (17)	$2\frac{1}{2}$–3 (3)
April 20	$4\frac{1}{2}$–15 (18)	4–8 (33)	3–$3\frac{1}{2}$ (2)
April 22	$3\frac{1}{2}$–10 (4)	$4\frac{1}{2}$–$6\frac{1}{2}$ (9)	2–$3\frac{1}{2}$ (7)
June 23	$3\frac{1}{2}$–$5\frac{1}{2}$ (9)	$3\frac{1}{2}$–5 (11)	—
June 24	—	5 (1)	—

Color

Color of body and appendages variable from one specimen to another, ranging from nearly transparent to yellow-orange and red. In the majority of specimens the color is rather uniform light yellow-orange over the entire animal; about 10%, however, have broad transverse bars or patches of red on the body and appendages that contrast sharply with the transparent or light yellowish-orange of the remaining areas. The eye is bright red. In alcohol-preserved specimens the large gnathopods are frequently medium to dark yellow, whereas other parts of the body are light yellow or whitish. Color pattern does not appear to be related to age, sex, locality, or host.

Range

Coastal waters of southeastern New England, U. S. A.; Long Island Sound, Vineyard Sound, Great Harbor in Woods Hole, passages between Vineyard Sound and Buzzards Bay, Cape Cod Canal; in water depths of 0.3 to 25 m. Has been found only in marine waters of full salinity (about 32‰).

Material examined

Holotype, adult male 15 mm. long, deposited in the U. S. National Museum (No. 113031). Allotype, ovigerous female 7 mm. long (USNM No. 113032). Paratypes, 17 males ranging in length from 4½ to 14 mm.; 32 females ranging in length from 4 to 8 mm.; and two juveniles 3 and 3½ mm. long. All type specimens were taken from a single *Asterias forbesi,* 7½ cm. radius. Collection was made April 20, 1965, at water depths of 10 to 15 m. in Vineyard Sound, 3 km. northeast of Nobska Point, off Falmouth, Massachusetts. Specimens were collected by John J. Valois and Paul Shave.

Quicks Hole (between Buzzards Bay and Vineyard Sound); water depth 8 m.; water temperature 3.9° C.; March 29, 1965; 35 specimens (6 males; 12 females, 7 ovigerous; 7 juveniles) on *Asterias forbesi.*

Quicks Hole; water depth 8 m.; water temperature 6.1° C.; April 22, 1965; 20 specimens (4 males; 9 females, 5 ovigerous; 7 juveniles) on *Asterias forbesi* and *Asterias vulgaris.*

Lagoon Pond, Martha's Vineyard (Vineyard Sound); water depth 0.3 to 2 m.; June 23, 1965; 20 specimens (9 males; 11 females, 10 ovigerous) on *Asterias forbesi.*

Great Harbor in Woods Hole; water depth 13 to 20 m.; water temperature 3.9° C.; March 25, 1965; one female on *Asterias forbesi.*

Vineyard Sound, 3 km. south-southeast of Nobska Point; water depth 22 to 25 m.; March 30, 1965; 37 specimens (17 males; 17 females, 16 ovigerous; 3 juveniles) on *Asterias forbesi.*

Vineyard Sound, 3 km. northeast of Nobska Point; water depth 9 m.; water temperature 3.4° C.; March 6, 1965; 12 specimens (8 males; 4 females, 2 ovigerous) on *Asterias forbesi.*

Cape Cod Canal, Sandwich; water depth 1 m.; June 24, 1965; one ovigerous female; on *Asterias vulgaris.*

(Long Island Sound specimens were identified by John C. McCain and were not examined by us. These specimens, numbering approximately 150, were collected by Clyde L. MacKenzie, Jr., from starfishes dredged at a depth of 10 m. off Pond Point, Milford, Connecticut).[2]

Commensal Relationship

C. grahami has been observed on two species of starfishes—*Asterias forbesi* (Desor) and *Asterias vulgaris* Verrill—both of which belong to the family Asteriidae. These species of *Asterias* are common in the inshore waters of southeastern Massachusetts (Sumner, Osburn and Cole, 1913; Galtsoff and Loosanoff, 1939). *C. grahami* has not yet been found on other organisms, such as algae or hydroids, with which caprellids are most commonly associated. This species clings to the starfish with the posterior pereopods in the manner characteristic of Caprellidae. The posterior three pairs of pereopods and pereods 6 and 7 lie flat against the starfish. The body bends sharply upward at the juncture of pereods 5 and 6, and the anterior section assumes various positions. The position most frequently observed is the body arched above the host; sometimes, however, specimens lie inconspicuously against the host's body between the rows of spines. When physically removed from the starfish they swim about by alternately bringing together the ends of the body and then straightening, all of which is accomplished quickly in short sequences. As many as 53 specimens of *C. grahami* were found on a single starfish (*A. forbesi*), the radius of which was 7½ cm. The density, however, varies considerably from one host to another.

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[2] Through the courtesy of J. C. McCain, this collection was subsequently examined by the authors.

This relationship appears to be very similar to that mentioned by Chevreux and Fage (1925) for *Pariambus typicus* (Krøyer). These authors reported that in European waters this caprellid occurs on *Asterias rubens* L. and *Solaster papposus* (L.).

Four specimens had a dense growth of filamentous green algae on the body and appendages anterior to pereod 5.

RELATIONSHIP OF C. GRAHAMI

Morphologically *Caprella grahami* appears to be most closely related to *C. unica* Mayer and *C. linearis* (Linnaeus). Although the latter species is larger, some of the appendages and body parts have similar shape and embellishment. Several characteristics of *C. grahami* that are useful for distinguishing it from *C. linearis* and other species are: its moderately stout body; pereods without knobs or tubercles on the dorsal surface; propodus of gnathopods 1 and 2 broad; dactyls of gnathopods and pereopods stout; absence of spines and scarcity of setae on posterior three pairs of pereopods; and the propodus of these pereopods with the palm margin not strongly concave. Also, in *C. grahami* the color is usually lighter and the pattern more uniform, whereas in *C. linearis* the color is usually darker and the pattern frequently streaked and speckled. *C. unica* is a moderately deep-water species known only from the type specimen, an immature male, collected south of Nantucket Shoals (Station 2253; 40°34′30″ N. lat., 69°50′45″ W. long.) by the U. S. Fish Commission in 1884. The stout dactyls on the gnathopods and pereopods, and the configuration of the propodus of gnathopod 2, are similar to those of *C. grahami*. *C. unica*, however, has numerous small and medium-sized spines on the dorsal and dorsolateral portions of the cephalon and pereods (Mayer, 1903).

The unique association of *C. grahami* with Asteriidae facilitates its collection and identification.

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A CONTRIBUTION TO THE SYSTEMATICS OF THE MARINE TUBIFICIDAE (ANNELIDA, OLIGOCHAETA)

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The brackish-water and marine species of Tubificidae were listed in an earlier publication (Brinkhurst, 1963a), but since then some new species have been described from North America (Brinkhurst, 1965; Brinkhurst and Gitay, in press). More recently, the acquisition of material from the Bahamas, Florida, and North Carolina has made possible the re-description of a species originally described by Pierantoni (1902, 1916) from Naples. Amongst the same collections were specimens of a new marine enchytraeid (to be described elsewhere by Dr. C. R. Kennedy), and specimens of a new tubificid. Material from brackish-water sites in Alaska is also to hand, but will be used as a basis for a separate revision of the genus *Monopylephorus* Levinsen.

The various species described by Pierantoni (1902, 1904, 1916) have seldom if ever, been reported by subsequent workers. In an attempt to trace material of these poorly-known species, I visited Naples in 1964. A careful search of mud-samples from the type localities failed to produce any new material, and the types were not found in the Marine Biology Station. The types may be in the museum of the University of Naples, but no response to appeals for material has been received. It was a considerable surprise to find material of what was clearly recognizable as *Heterodrilus arenicolus* Pierantoni among collections sent to me by Dr. Meredith Jones (The Smithsonian Institution), and Dr. J. H. Day (Duke University Marine Laboratory, Beaufort, North Carolina, and The University of Cape Town.)

Examination of whole-mounts, dissections, and sections made it clear that North American specimens shared some of the characters of *H. arenicolus* and *Clitellio subtilis* Pierantoni. Pierantoni (1916, p. 90) expressed the view that the differences in the genitalia which served to place these two entities in separate genera were, perhaps, misleading, in view of the close similarity of the rest of their anatomy. The discovery of spermathecae in my specimens, together with the re-description of the male efferent ducts of both this and *Clitellio arenarius* (Müller), make the retention of the genus *Heterodrilus* invalid. The genus *Limno-*

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driloides has already been merged with *Clitellio* (Brinkhurst, 1963b), and the new material shows some relationship to species formerly in that genus. The genus *Clitellio* may now be defined as follows:

Clitellio Savigny, 1820

Male efferent ducts tubular, prostate glands present or absent; when present, attached to atria by a broad base. Pseudo-penes or penes present. Spermathecae present, with spermatophores. No coelomocytes. All species thus far described lack hair setae and pectinate setae, and occupy brackish or salt-water habitats.

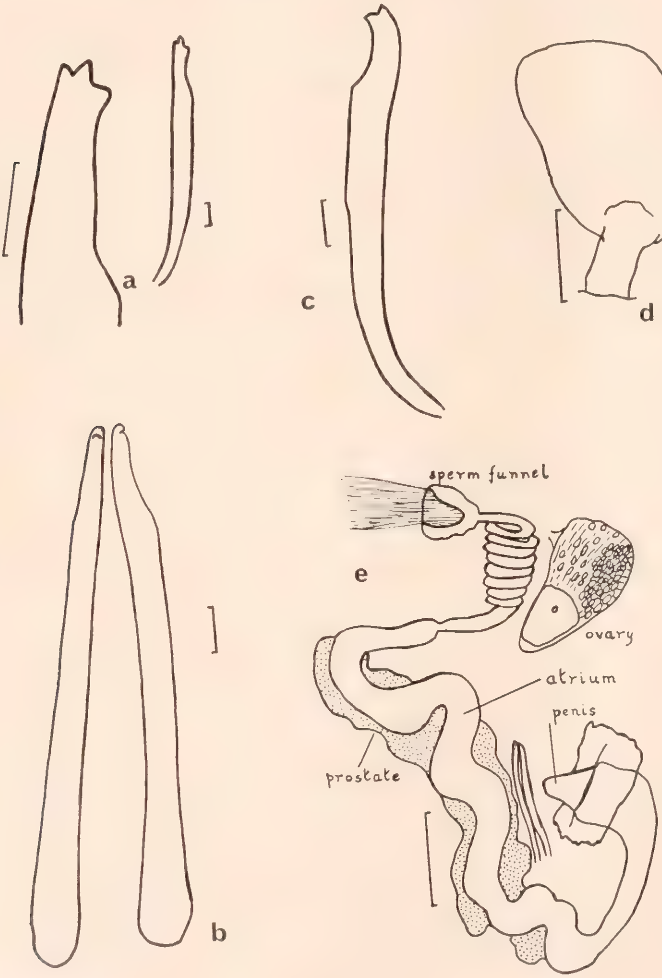

FIGURE 1. a, anterior trifid setae; b, penial setae; c, posterior bifid seta; d, spermatheca; e, male efferent ducts, showing coiled vas deferens, diffuse prostate, pseudo-penis, penial setae. An ovary is shown close to the coiled vas deferens. Scales a, b, c = 0.01 mm., d, e = 0.1 mm.

Type species: *Clitellio arenarius* (Müller, 1776)

The true nature of the male efferent ducts was not appreciated when the preliminary review of the Tubificidae was drawn up (Brinkhurst, 1963b). Subsequent examination of dissections and whole-mounts of *Clitellio arenarius* proved that the division of the atrium into two lobes is more apparent than real. The male ducts have to occupy part of the sperm-sac, owing to their length, and the atrium is constricted at the point at which it passes through Septum 11/12. The atrium is thin-walled, and there is no prostate gland.

The definition of the genus was revised later (Brinkhurst, 1965) when it was noted that no species thus far described had genital setae. The statement was carefully worded to indicate that the discovery of a species that appeared to fit the definition in all other respects would not be excluded from *Clitellio* simply by the possession of genital setae. The revised definition stated that pseudo-penes were present in the genus. Michaelsen (1908, 1914) claimed that true penes were present in *C. arenarius* and in *C. winckelmanni.* Pierantoni (1904, 1916) described penes in *Limnodriloides, Heterodrilus* and *Clitellio* species, some of which appear more like pseudo-penes in the illustrations. Examinations of sections of *C. arenarius* show that there are, in fact, very simple penes in the penis-sacs. The distinction between eversible pseudo-penes, protrusible pseudo-penes and penes, has been discussed elsewhere (Brinkhurst, 1966), where it was shown that in eversible pseudo-penes the muscular layer of the atrial wall became modified into two layers joined by fibers running across a space formed between the layers. This would allow the lining cells of the atrium to be protruded through the muscle layer, the protrusion being completed by the eversion of the in-tucked pouch of the body-wall which forms the penis sac when the penis is present. The true penis is then defined as the permanent lobe lying in this sac, penetrated by the thin distal end of the atrium. The penes of *C. arenarius* are of the simplest possible type, with the split developed in the muscular layer of the atrium, but there is also a small lobe of the penis-sac which looks as though it is permanent once formed. The true anatomical male pore is thus at the end of this small penis, and not at the summit of the eversible penis-sac. In *H. arenicolus* there do not seem to be any true penes, but the separation of the lining layer and muscular layer of the terminal part of the atrium is well-developed.

Clitellio arenicolus (Pierantoni, 1902)

Heterodrilus arenicolus Pierantoni, 1902, pp. 115–117, fig. 3; 1916, pp. 87–90, Pl. 4, fig. 6–11: Brinkhurst, 1963a, pp. 714; 1963b, pp. 74.

Length 0.75–1 cm., 45–60 segments. Prostomium elongate, concial. Anterior end of worm markedly narrow. Setae in four bundles per segment from segment II. Setae of segments II–IX, two per bundle blunt-ended or trifid (Fig. 1a). A single unmodified setae in each bundle of X, ventral bundles of XI each with two blunt penial setae each (Fig. 1b). Post-clitellar setae single, with short teeth, the upper tooth usually the shorter, or even rudimentary (Fig. 1c). Spermathecal pores in line with and posterior to the ventral setae of XI. Female pores in line of ventral setae in 10/11, spermathecae in X (Fig. 1d). Male ducts with coiled vas deferens, tubular atrium with prostate gland (Fig. 1e). Ejaculatory duct narrow, protrusible pseudo-penes. Spermatophores formed. No coelomocytes.

U. S. National Museum Collection: 33004, 0.5 km. N. entrance point, W. side N. Bimini (25°43′2″N, 79°19′0″W), August 27, 1962; 33009 off-shore Panama City, Florida (30°03′6″N, 86°08′8″W), April 8, 1958. Four slides. Coll. M. L. Jones.

J. F. Day Collection: Beaufort Shelf transect, off North Carolina.
34°36′5″N, 76°30′0″W, 5 m., depth, April 19, 1965;
34°34′5″N, 76°25′5″W, 20 m., April 19, 1965;
34°19′6″N, 75°56′8″W, 130 m., April 6, 1965 (12 specimens)

The male efferent ducts and spermathecae are almost exactly the same as those described for *C. subtilis* by Pierantoni (1916). The only exception is the presence of a prostate gland. This gland envelops most of the atrium, but does not appear to be a simple diffuse cellular layer of the form found in *Bothrioneurum, Monopylephorus, Rhyacodrilus,* and others. On the other hand, no distinct point of entry into the atrium could be discerned, so that the prostate does not appear to be of the bulky type found in the genera *Tubifex, Limnodrilus* and many others. It is just possible that it is intermediate in nature, being lobed but rather broadly attached to the atrium as in *C. appendiculatus* (Boldt, 1928), but this was impossible to discern on either the sections or dissections.

The tridentate anterior setae are identical to those described and figured for *H. arenicolus* by Pierantoni (*loc. cit.*). The penial setae are not smaller than the others (as in *H. arenicolus*, according to Pierantoni), but are larger, as in most tubificids with genital setae. The explanation of this apparent confusion may be attributable to faulty interpretation of poor sections in the original account, or simply to incomplete maturation of the male ducts in the Italian specimens. The absence of spermathecae in some specimens of otherwise normal species has already been noted as a rare variation in tubificid systematics. I conclude that *C. subtilis* is likely to prove to be no more than a variety of *C. arenicolus,* but the discovery of fresh material for comparison with forms bearing trifid setae must precede a final decision on the matter.

The second species to be described is attributed to a new genus. A single dissection revealed a unique type of male efferent duct, and this was subsequently confirmed by sections of a specimen which had previously been examined in Amman's lactophenol, a process which did not improve the quality of the material.

SMITHSONIDRILUS nov. gen.

Male efferent ducts tubular, opening into a common chamber in association with two paratria. Prostate glands cap the paratria. Spermathecae present, with spermatophores. No coelomocytes.

Type species: *Smithsonidrilus marinus* nov. sp.

Smithsonidrilus marinus nov. sp.

Length 10–15 mm. × 0.75 mm. 40–70 segments. Worm wound in a thin coil with long narrow segments. Prostomium short and blunt. Pharynx eversible.

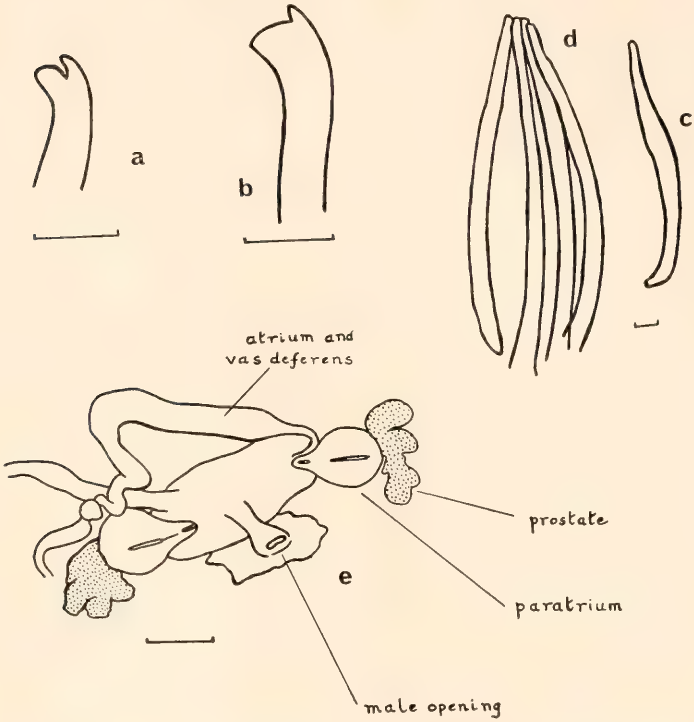

Figure 2. a, anterior seta; b, posterior seta; c, spermathecal seta; d, penial setae; e, male efferent ducts, showing median chamber with single median superficial male pore, two paratria with prostates, two vasa deferentia (incomplete). Scales a, b, c, d = 0.01 mm., e = 0.1 mm.

Setae all bifid crotchets, those of the anterior bundles 2–5 in number, with the upper tooth shorter and thinner than the lower (Fig. 2a), falling to 1–2 per bundle with even shorter upper teeth posteriorly, the lower tooth being exceptionally broad (Fig. 2b). Spermathecal setae blunt, 3–4 on each side (Fig. 2d). Vas deferens and/or atrium (scarcely distinguishable) short and thin, no prostates present on the atria. These ducts open near the base of the paratria, which project into a median chamber with a midventral superficial male pore (Fig. 2e). Paratria bear prostates. Spermathecae with spermatophores 10–15 times longer than broad.

U. S. National Museum Collection: Holotype 32967, Tampa Bay, Florida, 27°51′4″N, 83°35′4″ W, 27°51′3″N, 83°33′2″W, January 29, 1959; Paratypes 32968–71, Panama City, Florida, April 8, 1958; Pt. Boca, Ciega Bay, Florida, January 29, 1959; S. side Tavenier Key, Florida, July, 1960. Coll. M. L. Jones.

J. F. Day Collection: Beaufort Shelf transect, off N. Carolina, 34°34′5″N, 76°25′5″W, 20 m., April 19, 1965; 34°19′6″N, 75°56′8″W, 130 m., April 6. 1965.

The paratria of *Smithsonidrilus* are very similar to those of *Bothrioneurum*, in which tubular vasa deferentia and atria covered with diffuse prostate glands enter a median chamber into which these practically solid structures project. There are no spermathecae in *Bothrioneurum*, the spermatophores being attached externally, and there are coelomocytes, both in contrast to the new genus, which also lacks the glandular covering of the atrium. The prostate cells capping the paratria were overlooked in some descriptions of *Bothrioneurum* species, and were only seen here in the sectioned specimen. It is quite probable that these cells are readily detached from the paratria when these are dissected out of the worm. In the dissection, the efferent ducts did not appear to be associated with the paratria, but in the sections it seemed possible that the ducts became very narrow, running along the wall of the lower half of the paratria to open close to the hollow base of the latter. The paratria showed traces of a central canal in section and dissection, but there was no clear lumen except near the distal end (that opposite to the prostate cells, which is protruded in copulation in all probability). Penial and spermathecal setae were also only seen in the material from North Carolina, but these could easily have been lost in dissecting the single mature specimen from Florida.

There are some superficial similarities between this species and that recently described by Marcus (1965) under the name *Jolydrilus jaulus*. This Brazilian brackish-water species also has a median male pore, but has protrusible pseudo-penes at the end of short tubular atria. It has no spermathecae, and apparently lacks coelomocytes.

It is clear that there are more marine species to be described in this family, usually held to be almost exclusively fresh-water in habit. The small size of many marine oligochaetes in comparison to many other marine invertebrates probably ensures that either they are not obtained by the commoner sampling grabs employed in marine work, or they are overlooked in subsequent sorting.

The discovery of species known formerly from Naples in material from the Atlantic and Gulf Coast of North America further supports the view that many of these oligochaetes are widely distributed. The marine tubificid fauna of the New England states of the U. S. A. has already been shown to be rather similar to that of Europe and the Mediterranean, with certain exceptions, such as *Tubifex costatus*.

I must record my gratitude to Dr. Meredith L. Jones and Dr. John H. Day for the gift of material, and to Mr. C. A. Grainge for sectioning the worms.

SUMMARY

The re-discovery of a tubificid originally described as *Heterodrilus arenicolus*, together with a study of the efferent ducts of *Clitellio arenarius*, has led to the merging of *Heterodrilus* in *Clitellio*. *Clitellio subtilis* seems to be very closely related to *C. arenicolus*. A new genus is erected to receive a second marine species discovered, like the first, in material from the Gulf of Mexico and the Atlantic seaboard off North Carolina, U. S. A.

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BROODING BEHAVIOR OF A SIX–RAYED STARFISH, LEPTASTERIAS HEXACTIS[1]

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It has been known for a long time that certain starfishes, found in all three orders of the class, exhibit the habit of caring for their young. For example, Sars described the brooding habits of *Leptasterias mulleri* (Order Forcipulata) and *Henricia sanguinolenta* (Order Spinulosa) in 1846. Thomson observed the brooding habit of *Archaster excavatus* (Order Phanerozonia) in 1878.

Since then a number of brooding species have been added to the list. Ludwig (1903), Fisher (1940), and Hyman (1955) have named brooding forms and their distributions. It was pointed out by these authors that most of the brooding species are inhabitants of deep waters of the Antarctic and sub-Antarctic areas. However, some are commonly found on both the Atlantic and Pacific coasts of North America (Verrill, 1914; Fisher, 1911, 1928, 1930). Fell (1959) recorded a New Zealand shallow-water species, *Clavasterias suteri,* which is a brooding form also.

Brooding species usually produce a small number of large, yolky eggs, which undergo the direct type of development. Brooding species of the Orders Forcipulata and Spinulosa usually brood their young at the oral region by arching their arms to form a brooding chamber. However, in *Leptasterias groenlandica* (Lieberkind, 1920; Fisher, 1930), the young actually develop in the cardiac stomach. In the family *Pterasteridae* (Order Spinulosa), it was reported that the young develop in the nidamental chamber, the space between the aboral body wall and the supradorsal membrane (Koren and Danielssen, 1856; Fisher, 1940). Yet the evidence which I have gathered in *Pteraster tesselatus* of the San Juan Archipelago, Washington, indicates that this species does not in fact brood. The development is the direct type in this species, but is planktonic. Brooding species in the Order Phanerozonia, such as *Leptychaster almus* (Fisher, 1917) and *Ctenodiscus australis* (Lieberkind, 1926), usually brood their young among the paxillae on the aboral surface of the body wall. *Leptychaster* of Greenland (Order Phanerozonia), according to Fell (1959), hatches its young in the stomach, as does *Leptasterias groenlandica.*

All the publications of which I am aware that deal with brooding behavior in asteroids are limited to brief descriptions in connection with the studies of classification. No serious study on this subject has been reported. *Leptasterias hexactis* provides ideal material for such a study; it is an abundant intertidal species which makes it easier to observe in its natural environment, and when collected, its small size makes it easy to handle and observe in the laboratory. A number of observations on brooding specimens in their natural habitat, as well as in the laboratory, have been made, and a number of simple experiments have also been performed in the Friday Harbor Laboratories.

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[1] This work represents a part of a dissertation submitted to the University of Washington in partial fulfillment of the requirements for the Ph.D. degree granted in August, 1964. The author is greatly indebted to Dr. R. L. Fernald for his advice and encouragement. Present address: Department of Life Science, Sacramento State College, Sacramento, California.

General Observations

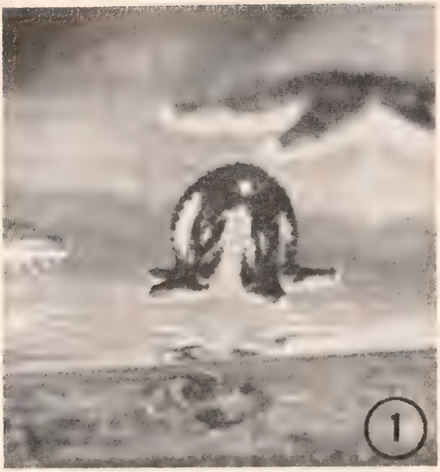
The animals in the Friday Harbor area enter the reproductive season at the end of November and terminate in April. The eggs are yolky and measure about 0.9 mm. in diameter. The number of embryos in each brood varies from 52 to 1491 and is correlated with the size of the female animal.

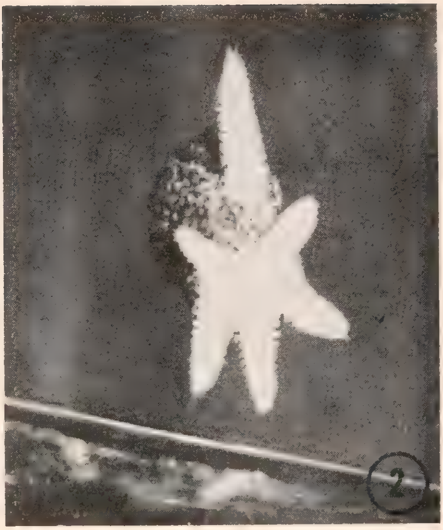
In order to examine the brooding habits more closely, four to five animals selected at random were kept in a small glass aquarium equipped with running sea water. When the females were ready to spawn, they assumed a typical brooding position (Figs. 1–3). If one was located on the wall of the aquarium, she arched her arms to form a brooding chamber and began to release eggs. During the first few minutes, the eggs were not sticky and did not adhere to each other. They were heavier than the sea water and tended to fall to the bottom of the aquarium. The female had to catch them by means of her tube feet and place them in the brooding chamber. Some eggs, especially those shed from the gonopores of the lower interradii, fell away. The males were spawning prior to the females or at the same time. At some times the spawning of male animals was unobservable and at others the spawning caused the water to become milky for a couple of hours. After the eggs were fertilized, the fertilization membrane became so sticky that the eggs adhered to one another and formed an almost solid mass (Figs. 4, 5). The process of shedding eggs lasted for several hours.

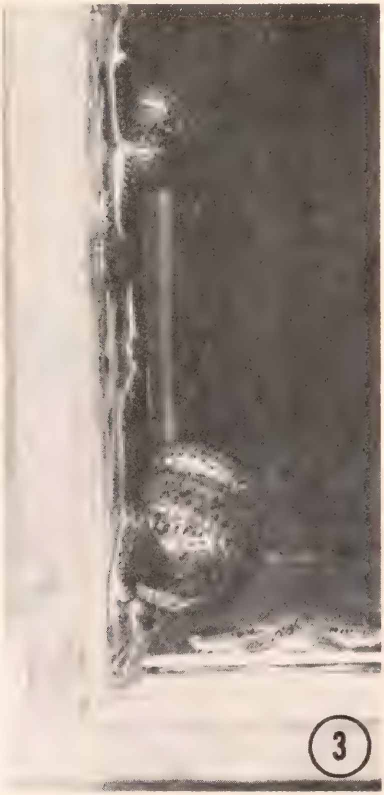
In the natural environment, the animals brood on the undersurfaces or sides of rocks. Attachment of the female to the undersurface has an obvious advantage during spawning, for in this position the eggs can be collected easily in the brooding chamber and the eggs always are provided with some moisture at low tide. In addition, direct exposure to sunlight is avoided. After spawning, the female starfish usually remains in this position until the young have completed metamorphosis. If the female is disturbed, she may move to another place, but does not give up her brooding activities.

In making collections, the animals were often placed in plastic bags containing sea water. These brooding animals usually brought their arms together to form an enclosed chamber, or lay free in the water or attached themselves to the plastic bag with the tube feet at the distal ends of the arms. They often remained in this position overnight without forsaking the embryos when the plastic bag was floated in the aquarium. After being released from the bag, they moved around in the aquarium and finally found a place to attach and resumed the brooding position again.

During the first 40 days of brooding, the position remained unchanged. The female attached herself only by the use of the tube feet at the distal end of the arms. The tube feet at the other parts of the arms were used to hold the embryos and orient them by pulling and twisting. The pressure exerted on the embryos by







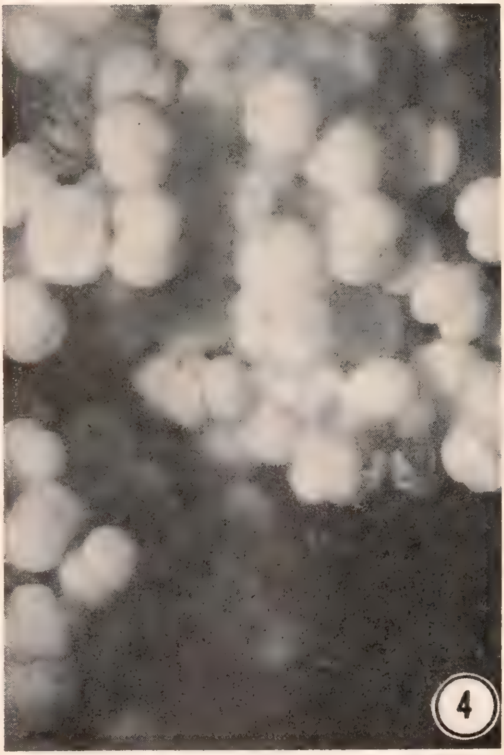
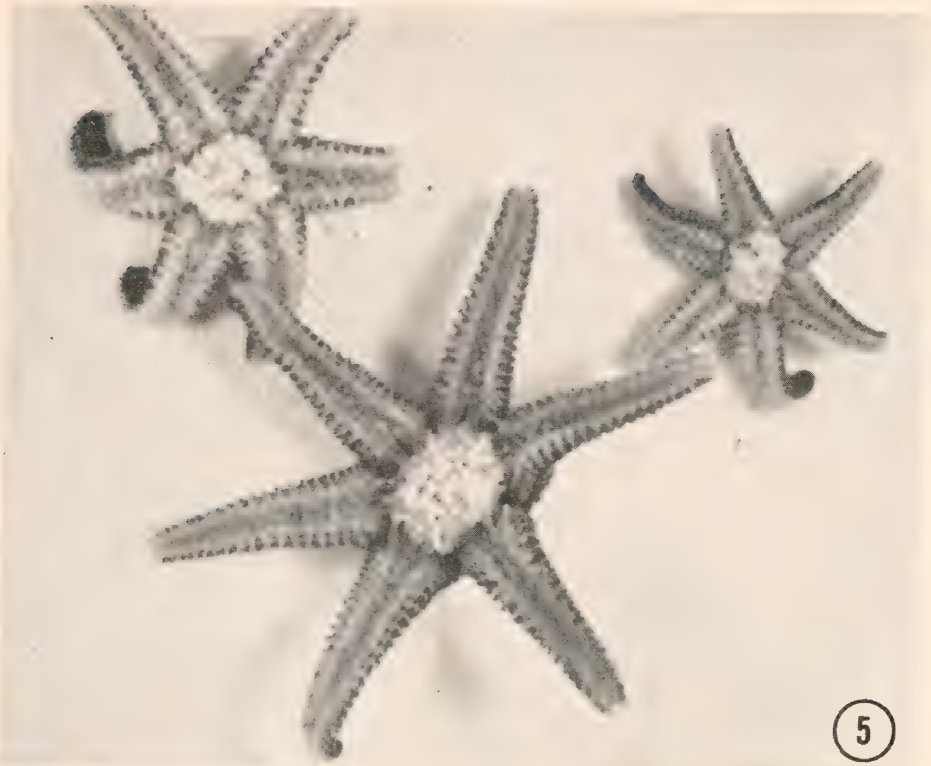


FIGURE 1. Lateral view of a brooding female on the wall of a Plexiglas tank. Natural size.

FIGURE 2. Oral view of a brooding female on the wall of a Plexiglas tank, showing the completely closed brooding chamber. Natural size.

FIGURE 3. Lateral view of two brooding females on the wall of a Plexiglas tank. Natural size.

FIGURE 4. Cleaving embryos; most are in four-cell stage. Living specimen. 14 ×.



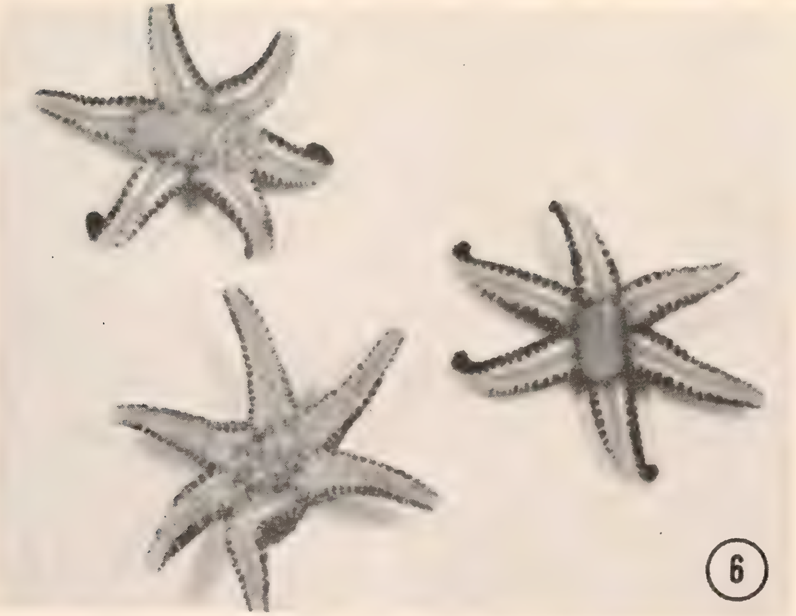


FIGURE 5. Three brooding animals turned oral side up, showing the application of the tube feet on the embryo masses. Living specimens. Natural size.

FIGURE 6. Oral view of three animals shown brooding artificial embryo masses made of gelatin capsules filled with wax. Living specimens. Natural size.

the tube feet may be fairly high and even temporarily distort the embryos. In most cases, from early development through metamorphosis, the embryos did not attach themselves to the substratum; instead, they adhered to each other and to the mother, and remained as a mass within the brooding chamber (Fig. 5). After about 40 days of development, when the tube feet of the young stars started to function, they began to attach themselves to the substratum but did not move away. It was at this stage that the adult resumed the flattened position; that is, she kept her arms straight, but remained with the brood. The whole picture reminds one of a mother hen protecting her chickens under her feathers and wings. After two months of development, when the preoral lobe of the young starfish had been completely absorbed, and the mouth opened and independent existence was possible, the mother animal left.

One female starfish was observed to spawn twice in the tank. This animal just moved a few inches away from her first brooding position and spawned again. The second spawning was about one month after the first and all of the first brood of embryos were metamorphosing and attached to the substratum. However, in most cases the female animals spawn only once per breeding season.

One can remove the embryos from the brooding chamber by a water current from a pipette. There are always small sand granules and debris which accumulate at the peristomial area, but the embryos in all cases observed were completely clean. This observation suggests that one of the functions of brooding is to keep the embryos clean.

CULTURE IN VITRO OF THE EMBRYOS

When the embryo masses were removed from the brooding chambers at the early cleavage stage, two methods of rearing them in the laboratory were repeatedly attempted:

(1) Ten embryo masses were placed in a small aquarium with a continuous flow of sea water. This method would simulate a situation in which the animals would not brood, but only spawn their gametes in the sea water without further care. None of the embryos treated in this manner survived more than five days; due to the sticky nature of the fertilization membrane, they were soon covered by debris and became infected by bacteria and protozoans, and the whole embryo mass decomposed.

(2) Ten embryo masses were placed in separate fingerbowls containing filtered sea water maintained at a temperature of 10° to 15° C. on sea water tables. The sea water in the fingerbowls was changed daily. The embryos seemed to develop satisfactorily until the postgastrula or even the early brachiolarian stage. However, the development of embryos in a mass was not synchronous. In addition, most of the embryos cultured in this way could not break through the fertilization membrane and hatching did not occur. These embryos soon were infected by bacteria or protozoans, and the cytoplasm became liquified and turned tea-brown in color. The embryos swelled to about twice normal size and finally burst. Occasionally, an embryo was able to get one brachiolar arm (usually the dorsal arm) of the preoral lobe out of the fertilization membrane, but only one or two per cent were capable of hatching, and then the process was much delayed.

By way of contrast, the development of the brooded embryos is synchronous and they have no apparent difficulty in hatching. They hatch simultaneously and the post-brachiolaria lasts four or five days until the first sign of metamorphosis. This suggests the possibility that the female may secrete some substances, possibly enzymatic in nature, which break down or weaken the fertilization membrane. To make a preliminary test of this possibility, some tissues of the peristome and cardiac stomach of a brooding animal whose larvae were at prehatching stage or just hatched were homogenized and this preparation was added to the dishes containing embryos at various stages of development. The embryos were observed under a microscope and allowed to develop. In no case was there any positive evidence that this juice weakens the fertilization membrane. It is thought that the hatching processes may be facilitated by the mechanical action on the fertilization membrane of the tube feet of the mother animal.

SELECTION OF SUBSTRATUM

The brooding animals not only favor the sides and undersurfaces of rocks, as stated above, but are also very sensitive to other characteristics of the rocks. Observations in 1960–1961 demonstrated that the brooding animals show preference for the dark and rough-surfaced rocks. Similar experiments were repeated in 1962–1963 with the same results.

Thirteen brooding animals were put on the floor of a small aquarium supplied with running sea water, in which there were two pieces of rock of comparable size, but one dark and the other pale. The results were recorded as follows:

Time	Dark rock	Pale rock	Wall of aquarium
0 hrs.	0	0	0
4 hrs.	6	0	7
24 hrs.	9	0	4

In another experiment all 13 animals were put on the pale rock and the results were as follows:

Time	Dark rock	Pale rock	Wall of aquarium
0 hrs.	0	13	0
1 hrs.	0	0	13
2 hrs.	2	0	11
5 hrs.	7	1	5
24 hrs.	9	1	3

The first experiment shows that 6 of the animals moved to the dark rock during the first four hours and 7 to the aquarium wall, but none of them moved to the pale rock. Within another 20 hours, about half of the animals originally settled on the aquarium wall had moved to the dark rock, but still none had moved to the pale rock.

The second experiment shows that all the 13 animals migrated to the aquarium wall from the pale rock in the first hour. Twenty-four hours later nine of them moved to the dark rock, one to the white rock and three remained on the aquarium wall.

In another aquarium two pieces of dark rock of similar size, one having a rough surface and the other smooth, were placed in with eight brooding animals. Twenty-four hours later seven animals were on the rough-surfaced rock and one on the smooth-surfaced one.

The non-brooding animals have been checked by the same method. They may show a preference for the dark and rough-surfaced rocks. However, their reaction is not as definite as that of the brooding animals.

FEEDING ACTIVITIES DURING BROODING

Since the embryo mass occupies the peristomial region of the brooding animal, whose tube feet are used in orientation of the embryos (Fig. 5), any feeding activity by the brooder would be handicapped. In fact, no feeding activities of brooding animals have been observed, either in the laboratory or in the natural environment. Is this non-feeding situation simply due to the blockage of the food passage or is it controlled by some physiological factors?

To investigate this problem a simple experiment was set up as follows: three small rocks, each covered with about a dozen barnacles (*Balanus glandula*), six limpets (*Acmaea digitalis*), six mussels (*Mytilus edulis*) and one chiton (*Tonicella lineata*), were placed in three separate aquaria, A, B, and C, and supplied with running sea water. In aquarium A five brooding animals were placed, the brood being about 10 days old. There were five brooding animals in aquarium B, but the embryo masses were removed from the brooding chamber. Five non-brooding animals were placed in aquarium C. Throughout a month of observation, no animals in aquarium A fed on the available food items; the animals in aquarium B did not feed in the first two days, but began to feed on barnacles on the third day; the animals in aquarium C started to feed within the first six hours.

This observation clearly indicates that these starfishes stop feeding only when they are actually brooding (aquarium A). This may imply that the cessation of feeding is either a simple matter of interference by the embryo masses or is regulated by physiological factors but a constant stimulation by the embryo is needed. The latent period of two days as shown by the animals in aquarium B may be the time required for readjustment after the embryos were removed.

It is of interest to note that for an average-size star (radius 2.5 cm.), it takes 24 hours to complete the feeding on an *Acmaea digitalis* (1.4 cm. diameter), 30 hours on a small *Tonicella lineata*, and 60 hours on a *Mytilus edulis*.

BROODING ON ARTIFICIAL EMBRYOS

If an embryo mass is removed from a brooding female for five hours and then returned, she will pick up the embryos and continue to brood. A brooding animal also will pick up an extra embryo mass if it is available. These observations lead one to believe that the animals actually recognize embryos of their own species.

To investigate this possibility an experiment was set up by placing 10 embryo masses together with 10 non-brooding animals in an aquarium. Within 8 hours, 9 of the 10 animals had picked up the embryo masses and settled on the aquarium wall to brood. Four of the 9 animals continued to brood until all the embryos completed metamorphosis; the other five ceased brooding activity during the first week. It was proved later by dissection that these five animals included three males (all spawned) and two females (non-spawned). The four which continued to brood were apparently unspawned females, for they also spawned their own eggs, and therefore possessed embryos of two different stages in the same brood.

The question thus arises as to how the animals recognize the embryo masses. In an attempt to answer this question the following four experiments were performed by using artificial embryo masses.

(1) Artificial embryo masses were made with wax and dyed with orange G. A piece of copper wire was inserted in the center of each of the wax blocks to keep it anchored at the bottom of the aquarium. The size, color, and shape of the artificial embryo masses were somewhat similar to the natural embryo mass. Ten of these artificial embryo masses were placed with 10 non-brooding animals in a small aquarium of running sea water; no response was observed in two days.

(2) The 10 artificial embryo masses prepared for this experiment were similar to those in experiment 1 except that they were punched with numerous holes by using a small needle and then soaked overnight in an homogenate of embryos of *Leptasterias.* It was hoped that some kind of substances from the embryos might be a factor in attracting the animals to brood. But after these artificial embryo masses were given to 10 non-brooding animals again, no reaction was observed in a two-day period.

(3) In a third experiment, 10 artificial embryo masses were made by filling gelatin capsules with colored wax and pieces of copper wire. After being placed in sea water, the gelatin swelled a little and became sticky; this condition is similar to that of the fertilization membrane. Two hours later, three of the ten non-brooding animals each picked up an artificial embryo mass to brood (Fig. 6). Twelve hours later, 8 of the artificial embryo masses had been picked up by 7 animals; one animal picked up two. The brooding of the artificial embryos, however, did not last more than two days. After 24 hours the gelatin capsules had swollen so much as to become loose from the wax blocks, and were discarded by the starfishes.

(4) For this experiment 10 non-brooding animals were given 10 artificial embryo masses which included five with gelatin capsules and five without gelatin capsules. Twelve hours later, three of those artificial embryo masses with gelatin capsules were picked up; none of the artificial embryo masses without gelatin capsules were picked up. The possibility that the animals were actually feeding on the gelatin capsules instead of brooding was ruled out by the fact that no extrusion of the stomach was ever observed in any of the cases.

These four experiments suggest that the animal's acceptance of a "mass" for brooding has little to do with its size, color, or shape. It also failed to provide evidence that chemotaxis plays a significant role in stimulating the recognition. Rather, the physical character of the surface layer of the embryo is possibly the main factor in its acceptance.

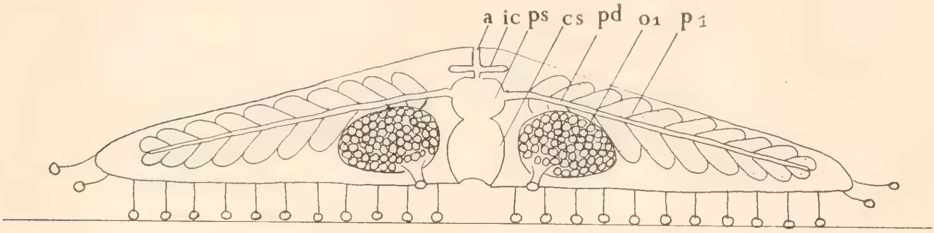
Conclusion and Discussion

In a recent study Arnold (1962) demonstrated that in the cephalopod, *Loligo pealii,* an artificial egg mass can stimulate sexual behavior, which is followed by the establishment of a social hierarchy. The stimulus, according to the author, is a visual response. On the other hand, Fell (1940), reported that in *Amphipholis squamata*, a brooding brittle star, there is actually a trophic relationship between the mother and the young. This is to say that the female animal secretes some nourishing substance(s) necessary for development of the embryos. In the present species, as it has been demonstrated, the animal will pick up an artificial embryo mass to brood, but this is apparently a response to the physical contact between the mother and the embryos. Meanwhile, no trophic relationship between the mother and young was indicated, because the embryos, after being removed from the mother, develop normally up to pre-hatching larval stage, and, if the fertilization membrane is removed manually, they will develop normally up to the young adult stage without any sign of morphological abnormality. In other words, the stored yolk in the ooplasm is sufficient to provide all necessary nutrients for development to a young adult. Thus, the major functions provided by brooding are protection, cleaning, maintenance of a uniform environment, and initiation of the hatching process.

Compared with all the other intertidal sea-stars at the San Juan Island area, *Leptasterias hexactis* has been very successful in spite of the fact that it produces only a small number of eggs and lacks the pelagic larvae to disperse the progeny. Embryos protected in the brooding chamber not only avoid exposure to severe effects of some environmental factors such as desiccation but also are safe from predators. The only predators for adult *Leptasterias* I have observed are other starfishes such as *Solaster*, *Crossaster*, and *Pycnopodia*. These were observed to prey on *L. hexactis* in the laboratory; however, species of these genera rarely exist together in the natural habitat.

The movement of cilia and tube feet is responsible for removing dirt and debris which become attached to the embryos. It is also responsible for maintaining a water current which insures a uniform environment and facilitates synchronous development. As for breaking through the fertilization membrane, there are two possible ways by which this may be achieved: (1) the embryo itself may secrete a digestive enzyme to dissolve the membrane; (2) the brooding animal may provide other chemical or mechanical means to rupture the membrane; possibly, a combination of these is involved. The failure of isolated embryos to hatch, as demonstrated in this study, may imply that the brooding mother is fully responsible for this process, but the possibility of the failure of embryos to secrete such a digestive enzyme because of the isolation from the mother is not excluded.

One of the most interesting problems, of course, is the control mechanism of brooding behavior. When an animal assumes a brooding position (Fig. 8) from a pre-brooding position (Fig. 9), one can easily see that at least three obvious changes occur in functional morphology. (1) Muscular system: the contraction of some muscles (longitudinal adambulacral muscles, for example) enables the animal to form a brooding chamber to house the embryos. This position lasts for at least 30 days. (2) Ovary: it is filled at the pre-brooding stage and emptied at the brooding stage, during which all the eggs are fertilized in the brooding chamber; they then form the embryo mass. (3) Pyloric caeca: materials such as lipids, polysaccharides, acidophilic granules, and zymogen granules, which are abundant in the epithelial cells of the pre-brooding animal, disappear after 30 days of brooding. Details of this phenomenon will be reported in a separate paper.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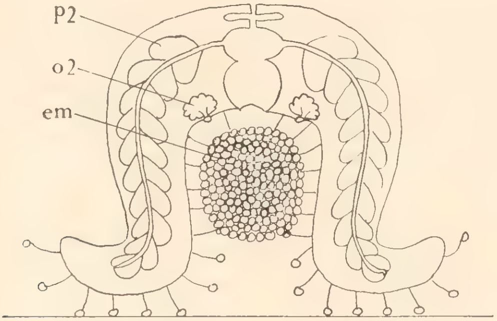

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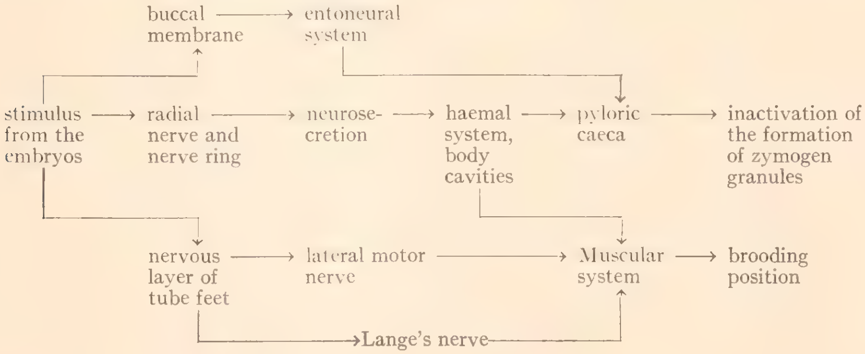
FIGURE 7. Diagrammatic drawing to illustrate a prebrooding animal (feeding). a. anus; ic, intestinal caecum; ps, pyloric stomach; cs, cardiac stomach; pd, pyloric duct; O_1, ovary before spawning; P_1, pyloric caecum of a feeding animal.

FIGURE 8. Diagrammatic drawing to illustrate a brooding animal. em, embryos; O_2, ovary after spawning; P_2, pyloric caecum of a brooding animal.

A number of other behavioral changes also occur during brooding, such as increased sensitivity to the substratum and cessation of feeding.

To maintain the brooding position, as shown in this study, a direct contact between the mother and the embryos is necessary, since the animal straightens her arms to a normal position and resumes feeding if the embryos are removed. Furthermore, the stimulus resulting from the contact between the embryos and the mother is thought to be physical rather than chemical in nature. But how does the stimulus pass from the contact area to the target organs? In other words, what is the coordination system? Taking the pyloric caeca and the muscular system as target organs, a hypothetic coordination process can be presented as follows:


buccal membrane
entoneural system
stimulus from the embryos
radial nerve and nerve ring
neurosecretion
haemal system, body cavities
pyloric caeca
inactivation of the formation of zymogen granules
nervous layer of tube feet
lateral motor nerve
Muscular system
brooding position
Lange's nerve

In support of this hypothesis, a number of facts, such as the nature of the stimulus and the changes in neurosecretion during brooding, have to be substantiated and studies along this line are being pursued.

Recent studies of Chaet (1964), Kanatani and Noumura (1962) and Noumura and Kanatani (1962) have shown that an intracoelomic injection of water extract from the radial nerve cord induces spawning in several species of asteroids. Furthermore, Unger (1960, 1962) has demonstrated the presence of neurosecretory products in the radial nerve of *Asterias glacialis* and their effect on motor activity, osmoregulation and pigmentation; therefore, it is reasonable to assume that neurosecretion may be involved in controlling the brooding behavior in the present species. A preliminary study on neurosecretion by histological methods in the present species revealed a scant number of neurons with secretory granules in the radial nerve cords, but it failed to demonstrate any substantial differences in quality between the brooding and non-brooding animals. However, the possibility that the brooding behavior is influenced by neurosecretion is far from exhausted.

SUMMARY

1. The brooding habit is apparently necessary for these animals; none of the embryos survived without brooding.

2. The main functions provided by brooding are protection, cleaning, maintenance of a uniform environment, and initiating the hatching process.

3. During brooding, the animals are particularly sensitive to the substratum. They not only favor the under and vertical surface of rocks, but also prefer the darker-colored and rougher-surfaced rocks.

4. At the time of brooding, *Leptasterias hexactis* stops feeding completely, but the animals will take food after a latent period of two days if the embryos are removed from the brooding chamber. Thus, a constant stimulation by the embryos is apparently necessary to prevent the animals from feeding.

5. During the breeding season, all the adult animals (female, male, spawned and non-spawned) are able to recognize their embryos and will pick them up to brood. This recognition is not due to the size, color, or shape of the embryo masses but rather to the physical nature of the fertilization membrane.

6. The significance and possible control mechanism of brooding were discussed.

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LIFE-HISTORY OF BOCCARDIA HAMATA (WEBSTER) ON THE EAST AND WEST COASTS OF NORTH AMERICA

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Polydora hamata Webster, 1879, was recently transferred to the closely related genus *Boccardia*, and *Boccardia uncata* Berkeley, 1927 and *Polydora uncatiformis* Monro, 1938 were placed in synonomy with *B. hamata* (Webster), a new combination (Blake, 1966). This species has been reported from a variety of muddy estuarine habitats on the west coast as *B. uncata* (Hartman, 1961) and as *B. hamata* (Blake, 1966). From Japan, Okuda (1937) collected *Polydora* (*Boccardia*) *uncata* ". . . from a muddy flat between crevices of rock." On the east coast of North America (Webster, 1879) and the Gulf of Mexico (Hartman, 1951; Rioja, 1960) this species has been recorded as *P. hamata* and has been found only in galleries of shells. Re-examination of the specimens identified as *Polydora* sp. (Dean and Haskin, 1964, tables 5 and 6) has shown them to be *B. hamata*. The specimens inhabited tubes of fine sediment on rocks between bases of the barnacle, *Balanus improvisus*.

In these two independent studies development was followed from eggs to young adults on the east coast and from the earliest planktonic larvae to young adults on the west coast. These two studies were synthesized and larval development from both coasts compared in the present paper.

We gratefully acknowledge the assistance of Dr. Keith H. Woodwick of Fresno, California, for identification of east coast adults, and to Mrs. P. A. Hatfield and Mrs. C. M. Blake for technical assistance.

MATERIALS AND METHODS

1. *East coast study*

Adults of *B. hamata* were found inhabiting burrows in shells of the hermit crab, *Eupagurus pollicaris* and in bottom samples of fine sand-shell mixture at 5 to 6 m. in one locality of the Mystic River. Adults for this study were obtained by cracking open hermit crab shells and examining the shell fragments with a dissecting microscope. A male containing motile sperm was obtained on April 2, 1963. On July 10, 1963, a partially exposed egg string was found in a burrow with an adult female *B. hamata* in the shell of *Lunatia heros*. The piece of shell with the partially exposed egg string was washed in several changes of filtered sea water. One sac was dissected from the string and placed unruptured in a separate dish so that development could be observed. Two more of the exposed sacs were ruptured during the following week to release some of the larvae for study. The rest of the string was left intact. Once or twice daily the dishes were examined for released larvae. Groups of five or more larvae of the same stage of development were placed in rearing vessels and fed a suspension of liver-powder—once a week for young stages, up to three times a week for late stage larvae. Rearing techniques have been described previously (Dean and Hatfield, 1963a; Dean, 1965; Hatfield, 1965). Specimens from the above source were maintained in laboratory culture until October 2, 1963.

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The larval study was a portion of a thesis submitted in partial fulfillment of the requirements for the degree of Master of Arts in the Department of Biology at Fresno State College. This study was in part supported by research grant GB13990 from the National Science Foundation, under supervision of Dr. Keith H. Woodwick.

Larvae were examined periodically under phase optics using either hanging drop or Saran Wrap techniques (Dean and Hatfield, 1963b). The accompanying Figures 1–5 are composite drawings made from photographs, sketches and descriptions.

Once the larval development of *B. hamata* was known, additional larvae were obtained from plankton samples for studies on metamorphosis and larval behavior. Plankton tows, taken at approximately weekly intervals throughout the year, have yielded larvae of this species from late June to late September. They have been most abundant, however, in August.

2. *West coast study*

Plankton samples were taken in Morro Bay at monthly or more frequent intervals from May 16 to September 3, 1964. *Boccardia hamata* larvae were encountered in all samples but were most abundant during June and July. Living material was brought to Fresno (a distance of 175 miles from the coast) in an insulated ice chest. In an air conditioned laboratory the larvae were sorted as soon as possible and placed with sea water in 60-mm. or 90-mm. diameter plastic Petri dishes or in battery jars with an aerator. Larvae survived better in the dishes. The larger dishes contained three compartments and were used for comparative studies of the influence of various substrata on settling and metamorphosis. A different type of substratum was put in each compartment and enough sea water added to allow larvae equal access to all substrata. The larvae were fed cultures of *Dunaliella*, dried and pulverized macroscopic alga, *Tethys* eggs or dead copepods from plankton samples.

Larvae were observed with a phase-contrast microscope. Most were placed on slides and covered with either Saran Wrap (Dean and Hatfield, 1963b) or coverslips. Some were observed in depression slides to allow greater freedom of movement. Photographs were taken with Polaroid and 35 mm. camera attachments. Figures 6 and 7 were prepared from photographs, sketches and notes.

Boccardia hamata adults were obtained from the silty mud of Morro Bay during Dec., 1963; Sept. and Dec., 1964; and Jan. and Feb., 1965. Neither ovigerous females nor egg capsules were collected; however, a male obtained in January of 1965 contained sperm.

Results

1. *Development of east coast larvae*

Larval development of *B. hamata* on both coasts was found to be alike in almost all details. A few significant differences have been discovered and are discussed in the next section.

The moniliform egg string within the *Boccardia* burrow was similar to that figured for *Polydora ciliata* and *Polydora hoplura* (Wilson, 1928). Each sac in the exposed portion of the string contained 10 to 12 moving embryos. The number of sacs hidden within the burrow was not determined.

Trochophores were released from the ruptured sac on July 10. At this stage the prototroch completely encircled the anterior end and a large area on the ventral surface was ciliated. By the following day one pair of black eyes and a telotroch had developed (Fig. 1). An apical tuft was not observed at any stage. By July 13th larvae in the sacs had developed to a 3-setiger stage with melanophores, prototroch, telotroch and two pairs of eyes. All segments had long capillary serrate swimming setae, those of the first being slightly longer. Although gastrotrochs, neurotrochs and nototrochs were not observed, the larvae were capable swimmers when removed from the sac. By July 15th the larvae had developed to a stage with four setigerous segments and one non-setigerous segment (Fig. 2). A nototroch was present on setiger 3 and a neurotroch extended posteriorly from the mouth through setiger 1.

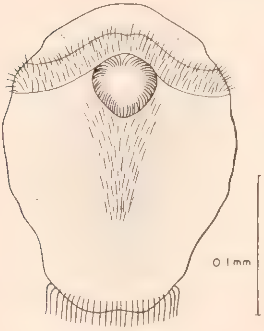

FIGURE 1. Trochophore in ventral view.

On July 18th almost all larvae emerged from the remaining sacs to begin their pelagic life. At this time the larvae had six setigerous segments and one non-setigerous segment anterior to the pygidium. These larvae were similar to the stage shown in Figure 2 except for size, the presence of gastrotrochs on setigers 3 and 5 and additional nototrochs on setigers 4 and 5.

The most obvious characteristics that serve to identify all pelagic stages of this species are the pigment patterns and general shape. Details of these and other morphological features are given below.

Pigment distribution is arranged in a definite pattern except for minor variations between individuals. Dorsally all segments have one pair of lateral stellate melanophores. A lateral process of the melanophores forms a small patch of pigment at the base of nearly all notopodia. Often setigers 1 and 5 lack these patches. All but setiger 2 have additional dorsal pigmentation. On setiger 1 a heavy transverse band of black pigment extends across the anterior edge of the segment and coalesces with the lateral stellate chromatophores (Figs. 3 and 4). This concentration of pigment causes setiger 1 to appear darkest, when viewed dorsally, on all larvae up to the 19-setiger stage. On the anterior edge of setiger 3 there is a transverse band of pigment composed of two melanophores that have usually coalesced medially. Near the posterior edge of this setiger are two smaller melanophores on either side of the midline. From setiger 4 posteriorly, paired patches of pigment are found near the midline and near the posterior edge of each segment. The latter pigment patches appear, under high magnification, to be composed of hollow black spheres (Fig. 2). Collectively, the spheres give a black coloration (Figs. 3, 4, and 5). Two concentrations of black pigment appear dorsally on the pygidium. The pygidium has a granular brown hue which is broken by a transverse clear band caused by the unpigmented telotroch cells. The anterior ring of the prostomium is usually dusky brown and deepens in hue medially in swimming-crawling stages.

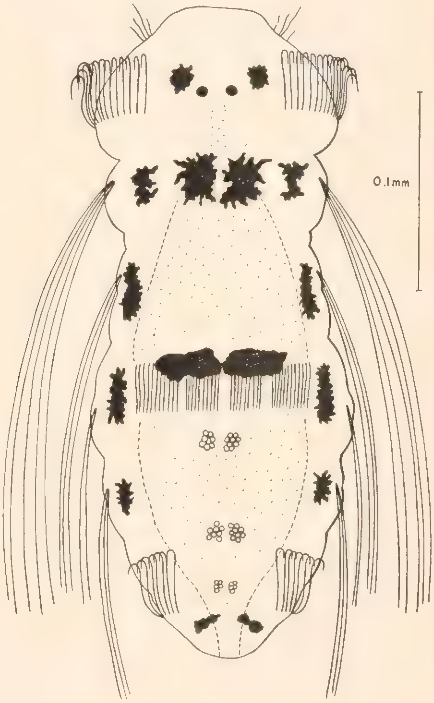

FIGURE 2. Four-setiger pre-pelagic larva in dorsal view.

The most obvious ventral pigmentation is a longitudinal bar of black pigment on each lateral lip. These bars are so dense and large that their location can be seen through the larvae in dorsal view (Fig. 3) and in lateral view (Fig. 4). The remaining ventral pigmentation is usually located on the extreme posterior edges of the segments beginning with the third setiger (Fig. 4). On some specimens

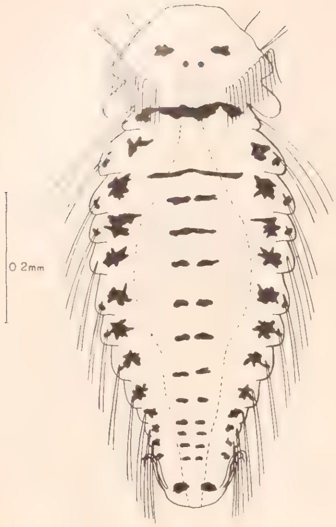

FIGURE 3. A pelagic 12-setiger larva in dorsal view. The thirteenth segment has been formed but its setae have not been secreted. Nototrochs and neurosetae have been omitted.

pigmentation also occurs on the anterior border of about setigers 4–7. Although the density of pigmentation here is highly variable between specimens, the pigment is usually arranged in two thin transverse stellate melanophores.

All pelagic stages are more or less fusiform in outline, regardless of whether they are viewed dorsally, ventrally or laterally.

Two pairs of eyes are almost always found on the larvae and are arranged as shown in Figures 2 to 5. On occasion, the larger and more anterior pair of eyes is divided giving the overall appearance of 3 pairs. At about the 13-setiger stage a cephalic crest or caruncle is formed (Fig. 4). By the 17-setiger stage the crest reaches the posterior edge of setiger 1 and the medial pair of eyes is high up on the crest.

Palps are first recognized in the 6- to 8-setiger larva as buds just anterior to the bases of the swimming setae of setiger 1. In the 13-setiger larva they are well developed and extend ventrolaterally to about the third segment (Fig. 4). In the

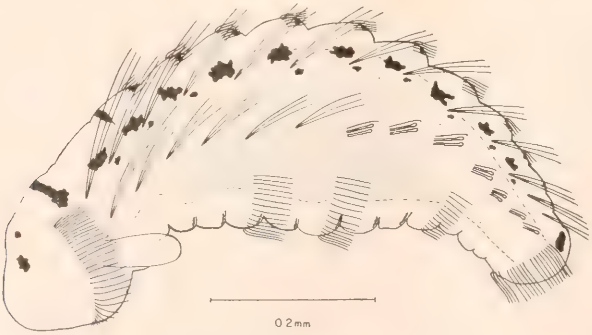

FIGURE 4. Thirteen-setiger pelagic larva in lateral view.

swimming-crawling stages the palps are extended while crawling (Fig. 5) or are tucked ventrally under the body while swimming.

The prototroch consists of a single row of stout cilia that passes laterally around the head of the larva from a point just anterior to the bases of the swimming setae of setiger 1 to the lateral lips. At the lips the prototrochal cilia grade into the shorter cilia that cover the oral region. The prototroch persists through the swimming-crawling stages and has been observed on some worms even after the larva has built a tube from sand grains. The telotroch consists of five indistinctly separated patches of cilia: one patch midventrally, two ventrolaterally and two dorsolaterally (Figs. 3 and 4). Gastrotrochs occur on setigers 3, 5, 7, 10, 13 and 15. The first gastrotroch is least developed. The remainder have longer cilia and extend across the ventral surface to the ventrolateral aspects of the body. Loss of gastrotrochs begins anteriorly with the disappearance of the first by the 13-setiger stage and the second by the 14-setiger stage.

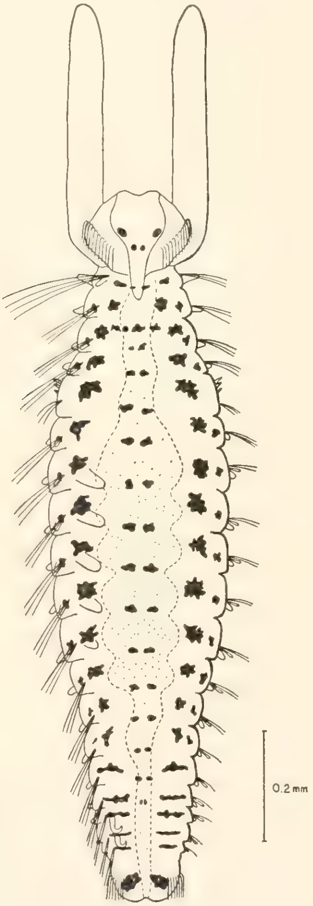

Figure 5. Nineteen-setiger swimming-crawling larva in the crawling position. Dorsal view. Neurosetae have been omitted on the left side. Notosetae and branchiae have been omitted on the right side.

Nototrochs, composed of four patches of cilia, make their first appearance on the third setigerous segment in the 4-setiger stage (Fig. 2). As a larva develops, prominent nototrochs appear on all setigers from 3 through 15. On late swimming-crawling stages much shorter cilia may also be observed on setigers 1, 2, 16, 17 and 18. Those on 1 and 2 are probably the beginning of nuchal ciliation while those on the posterior segments are probably the start of post-larval dorsal ciliation.

A poorly developed neurotroch extends posteriorly from the mouth through setiger 2. No ciliated pit was observed.

Sensory cilia are found on the anterior aspect of the prostromium.

All setae are capillary and serrate on larvae emerging from the egg sacs. The bases of the noto- and neurosetae are so close together that the larvae appear uniramous. During pelagic life the larval swimming setae are gradually replaced by non-serrate capillary setae and the distinction between noto- and neurosetae becomes more discernible. Hooded, bidentate crotchets first appear in the neuropodia of setiger 7 and are accompanied by curved, capillary setae. At the 15- to 19-setiger stage the slightly curved tips of the modified setae of setiger 5 begin to protrude from the body wall. The capillary noto- and neurosetae of this setiger are not as well developed as on adjacent setigers (Fig. 5). At the 19- to 20-setiger stage, a stout hooked seta accompanies the capillary setae in each notopodium of the posterior region (Fig. 5). In the east coast study the most anterior setiger bearing notohooks varied from 17 to 21.

Branchiae arise as outgrowths of the dorsal wall medial to the notosetae. These buds are not apparent until about the 15- or 16-setiger stage. By the 18-setiger stage, branchiae are conspicuous on setigers 7 through 11; those on 7 to 9 are well developed. Branchiae on segments anterior to the modified fifth setiger (a characteristic of the genus) do not appear in this species until sometime after the larvae have settled and metamorphosed into young adults.

At about the 19-setiger stage, the larvae begin a swimming-crawling phase. During this phase a larva will sink to the bottom of the vessel, lengthen and crawl over the bottom with a serpentine motion, then contract to the typical fusiform shape of the pelagic larva and resume swimming. At this stage the dense dorsal pigment of setiger 1 begins to break up and become rearranged as discussed later for the young adult.

Metamorphosis occurs at the end of the swimming-crawling phase when the larva loses its ability to resume swimming. In general, the amount of time spent on the bottom increases with increasing size. However, the stage at which individuals metamorphose varies greatly. The external morphological changes occurring at metamorphosis take several days to complete. Lengthening of the palps, loss of the telotroch and gradual reduction of the prototroch are the most apparent changes.

The rate of development of *B. hamata* larvae in the laboratory is shown in Table I. All observations were on specimens obtained from the egg string discovered on July 10, 1963. July 18, the day when the larvae were released naturally from the egg string, is taken as the beginning of pelagic life. The rate of development was relatively uniform between individuals for about the first three weeks of pelagic life. By the end of this time the majority of larvae had attained the 19-setiger stage. After the three-week period, the rate of development became more

Table I

Rate of development of Boccardia hamata larvae liberated from the same egg string

Date	Stage or setiger No.	Days after liberation
7-10-63	Trochophore	
7-11	Late trochophore	
7-13	3	
7-15	4	
7-18	6	0
7-19	6–7	1
7-23	8	5
7-24	9	6
7-29	13	11
7-31	12–16	13
8-5	19	18
8-6	17–19	19
8-11	15–20	24
8-12	23	25
8-14	18	27
8-23	23	36
8-24	22–30	37
9-18	19–27	62

variable. Length of pelagic life of east coast forms is estimated to be two and one half to three weeks.

2. *Development of west coast larvae*

The foregoing account agrees with observations on west coast larvae except as noted below.

1) Differences in pigmentation: The dorsal pigment pattern includes a transverse band on the second setiger (Fig. 6). This band makes setigers 1, 2 and 3 appear

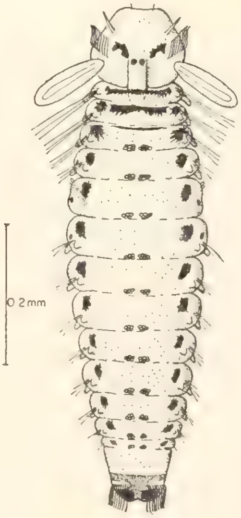

Figure 6. A pelagic 13-setiger larva from the Pacific coast in dorsal view. Nototrochs, gut and neurosetae have been omitted.

dark as opposed to only the first on east coast larvae. The pigment on the vestibule is never as strongly developed as on east coast forms. It is usually restricted to small black patches near the posterior borders of the vestibule. Ventral pigment on the body is often more strongly developed on west coast larvae and is usually restricted to setigers 3 to 7. Pigment is present in the intersegmental regions as shown in Figure 7.

2) Differences in ciliation: Gastrotrochs were not observed on setiger 3.

3) Differences in metamorphosis: Metamorphosis of west coast forms occurred very readily. Larvae usually metamorphosed between the 16- and 19-setiger stage, depending upon the substratum. In a few instances larvae with as few as 14 setigers metamorphosed. No larvae ever lived beyond 19 setigers without metamorphosing.

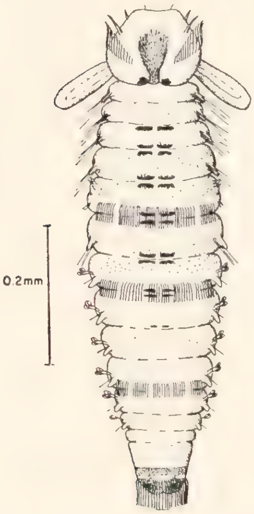

FIGURE 7. A pelagic 13-setiger larva from the Pacific coast in ventral view. Gut and notosetae have been omitted.

Mixtures of estuarine mud and open beach sand, ground coralline algae, and even planktonic detritus were used by the worms to construct tubes. Although larvae showed no preference for different substrata, growth after metamorphosis was fastest in those worms placed in estuarine mud. Following metamorphosis some specimens grew to over 40 setigers within three weeks.

During metamorphosis the segments lose their larval setae, leaving only those of the adult. The metamorphosed form is thinner and less fusiform than the pelagic larva. The head region and prostomium elongate and the latter becomes bilobate. The medial dorsal pigment bands on setigers 1 and 2 begin to shift forward and break up as the head narrows and elongates. The ventral pigment persists in its basic pattern through metamorphosis. Notosetae are lacking on setiger 1. A pair of fully developed modified setae with two bilimbate companion setae are present on each side of setiger 5. The pygidium elongates and small terminal processes possessing sensory cilia are evident for the first time.

3. Post-larval development

Post-larval development was best followed in west coast forms, due to their ease of metamorphosis and rapid growth. In specimens of 19 to 25 segments the anterior dorsal pigment pattern changes, forming an elongated mass of pigment on the caruncle posterior to the eye spots. Lateral to this a V-shaped pattern of pigment extends from the middle of segment 1 to the posterior margin of segment 2. This peculiar pattern begins to disappear at about the 34-setiger stage. The remaining dorsal pigment persists in its basic pattern only to about the 25-setiger stage, after which the inner rows of pigment become faint while the outer rows break up and disperse into small concentrated patches. On the 42-setiger worm, only an occasional patch remains, some near the notopodial lobes and others in the median region. This condition was also found in an adult specimen of 56 setigers taken from the shell of a hermit crab on June 28, 1961, from Cayucos, California.

The ventral pigment pattern persists, though faint, as late as the 42-segment stage.

Heavy, falcate hooks appear in the notopodia of posterior segments of either swimming-crawling stages or recently metamorphosed forms. As the worm grows, notohooks are formed in the newer segments and are lost in the older segments. For example, a 21-setiger specimen from the west coast had hooks on setigers 15 to 18, while on a 34-setiger specimen, hooks were present on setigers 26 to 32. Mature adults of 150 setigers have only 25 to 30 pairs of hooks in posterior segments.

The first branchiae to arise appear on setiger 7 as mentioned previously. As a worm grows, more branchiae are added posterior to setiger 7 and continue to the mid-region of the body. The appearance of branchiae on setigers 2, 3 and 6 does not occur until approximately three weeks after metamorphosis has been completed, regardless of the size of the specimen. For example, on the east coast, a 23-setiger worm that had metamorphosed three weeks previously had branchial buds on setigers 2, 3 and 6; a swimming-crawling larva of 27 setigers that had been maintained without sediment had no branchial buds. Although west coast post-larval forms grew much more rapidly than their east coast counterparts, the appearance of branchiae was also age-dependent. Branchial buds appeared on the anterior segments of a 42-setiger specimen three weeks after metamorphosis.

As the organism develops the prostomium becomes more flaring anteriorly. A deep median longitudinal groove appears on the dorsal side, its sides thicken and the groove becomes a narrow slit. Although the time for its formation varies, the slit is usually complete by the 42-setiger stage. At about the latter stage the caruncle extends posteriorly to setiger 3, and rapidly beating short cilia can be seen on the dorsum lateral to the caruncle.

Small processes develop on the dorsal aspect of the two rounded lobes of the pygidium. Each process bears sensory cilia.

The growth rate of west coast post-larval stages was variable and was, at least in part, attributable to the type of substratum offered. Unwashed but previously air-dried sediment taken from the locality of adults in the estuary supported the best growth in the laboratory. Air-dried sediment mixed with open beach sand supported the poorest growth.

4. *Metamorphosis experiments*

During the summers of 1963, 1964 and 1965, several experiments were conducted to determine whether sediment of different sizes and from different sources, pieces of or entire snail shells, or sediment and shell together would hasten metamorphosis over that of larvae maintained in culture vessels. Data from a representative experiment are presented in Table II. In summary, larvae provided with sediment occasionally built tubes of sand within a few days. Usually these tubes were only temporary, for a larva would leave its tube, swim again, then settle and construct another tube. Larvae that settled in sediment seldom lived for more than a week after metamorphosing. On the other hand, those kept without sediment delayed metamorphosis, but once metamorphosis did occur, the young adults usually survived for a month or more. One larva, 62 days after its release from the egg mass, had attained 27 setigers in the absence of a substratum and still had not metamorphosed.

Recently, Meadows (1964) has shown that sediment that has either been washed with distilled water or air-dried becomes unattractive to two species of the amphipod, *Corophium*. Since all sediment used in the above east coast experiments during 1963 and 1964 had been both washed in distilled water and air-dried, metamorphosis experiments were repeated during the summer of 1965 using untreated surface sediments (fine sand rich in detritus) from subtidal areas of the Mystic River. Settlement and metamorphosis were equally poor in these experiments as those with treated sediment.

Table II

Metamorphosis of Boccardia hamata larvae on different substrates

Vessel	Treatment	Observations											
4 larvae each	7-31-63	8-5	8-7	8-11	8-15	8-17	8-19	8-23	8-30	9-6	9-11	9-13	9-18
A	Sed. + shell	4s + tubes	3s	1 in Sed. 2s	2s	1s	1s	0	0	0	0	0	0
B	Sed.	4s + tubes	4s	4s	4s	1s 1 in Sed.	0	0	0	0	0	0	0
C	Shell	4s	4s	2s	2s	2s	1s	1s	1m	1m	1m	1m	1m
D	—	4s	4s	4s	4s	4s	4s	4s	4s	3s	2s	2m	1m

Sed. = sediment; s = swimming larvae; m = metamorphosed larvae.

An untreated *L. heros* shell that had previously contained a hermit crab was placed in a fingerbowl with filtered sea water and 10 late-stage *B. hamata* added. When examined after 7 days, two larvae had metamorphosed successfully on the shell's exterior and all but one pelagic larva had disappeared. Each metamorphosed larva had constructed a tube of silt-sized particles in a crevice, one in a growth line and one in a suture. When the tubes, which protruded from fresh excavations in the shell, were carefully dissected away, the excavations were already large enough to conceal the entire worm. On the 18th day of the experiment the shell was cracked open and the fragments examined. The two tongue-shaped burrows were approximately 0.5 mm. deep. No other *B. hamata* was found; however, one adult *Harmothoe imbricata* had been living inside the shell and may have eaten some of the settling larvae. It should be mentioned here that *H. imbricata* has been reared from trochophore to adulthood, the post-larvae thriving on a diet of polychaete larvae and copepods (Dean, unpublished).

Further studies on larval behavior and relationship to substrate are in progress and will be published later.

Discussion

Pelagic larvae of *Boccardia hamata* are most easily distinguished from other polychaete larvae in the plankton on the basis of pigment pattern and body shape. Reference to the morphological details given here can serve to confirm the identification. When examining a plankton sample from the east coast under a dissecting microscope, larvae of *Polydora ciliata* and *P. ligni* are the ones most likely to be confused with *B. hamata.* Larvae of both *Polydora* species are generally more slender than those of *B. hamata,* but overlap in size and shape has been observed occasionally. Although the dorsal pigment pattern of the three species is similar, at least under low magnification, *B. hamata* larvae are readily separated from the *Polydora* species by the pigmentation of the lateral lips. On *B. hamata* the bars of dense black pigment are longitudinal while those on these two species of *Polydora* are transverse.

On the west coast, *B. hamata* larvae are most easily confused with larvae of *B. columbiana, B. proboscidea, B. tricuspa* and *Pseudopolydora kempi.* The three *Boccardia* species differ in having a single dorsal row of chromatophores. *B. columbiana* and *B. proboscidea* have bushy-topped modified setae in setiger 5, while *B. tricuspa* has modified setae with three cusps. *Pseudopolydora kempi* larvae have a dorsal pigment pattern similar to *B. hamata,* with medial and lateral rows of chromatophores. But *Ps. kempi* has no ventral pigment and the dorsal medial chromatophores are heavy and not composed of small circles of black pigment. Further, its fifth setiger has fully developed parapodial lobes.

Hartman (1941) has figured the larvae of *B. proboscidea* and Rioja (1939) has illustrated *B. tricuspa* larvae. *Pseudopolydora kempi* and *B. columbiana* larval development and supplementary data on *B. proboscidea* will be reported later by Dr. Keith H. Woodwick.

B. hamata larvae are planktotrophic once they are released from the egg sac. In plankton samples they attempted to ingest almost any object in their path. In laboratory culture they ingested liver powder so readily that their mid-sections became swollen, making their fusiform shape even more pronounced. Plump larvae as well as the more slender specimens so easily confused with *Polydora* were observed in plankton samples.

Although the 6-setiger stage is considered to be the first pelagic stage, larval stages as early as trochophores were removed from the egg string and appeared to develop normally. Specimens of *B. hamata* larvae younger than 6-setiger larvae were not recorded from the plankton during more than two years of study.

Hannerz (1956) found that in most spionid species the first segment to bear branchiae in the larva corresponds to the most anterior setiger bearing branchiae in the adult. He noted exceptions that occur in *Spio, Microspio* and *Pygospio.*

In the former two genera, branchiae appear first on the segment coresponding to the second branchial segment of the adult while in the latter, branchiae are lacking during the entire pelagic life and the first part of benthic life. With respect to the appearance of branchiae, *Boccardia hamata* occupies an intermediate position between *Pygospio* on one hand, and *Spio* and *Microspio* on the other. On adult *B. hamata* branchiae occur on setigers 2, 3, 6, 7, etc., while on its pelagic larvae neither branchiae nor branchial *anlage* are observed anterior to setiger 7. Branchial *anlage* appear on setigers 2, 3 and 6 approximately three weeks after metamorphosis has been completed. The appearance of anterior branchiae is dependent on age past metamorphosis regardless of the size of the young worm.

During the course of these investigations, several differences between east and west coast populations of larvae and adults have become apparent. While the pelagic larvae from both coasts are nearly identical morphologically, differences exist in behavior during the periods of larval settlement and metamorphosis (Table III). Larvae from west coast populations settle and metamorphose readily on a variety of substrates while those from the east coast seem to be much more selective in choosing a substrate and can delay metamorphosis for extended periods. These behavioral differences are probably associated with habitat differences of the adults on the east and west coasts. Despite numerous benthic surveys by many workers on the Atlantic and Gulf coasts, there have been only a few records of this species and until now it has only been reported from galleries of shells (Webster, 1879; Hartman, 1951; Rioja, 1960). In the Pacific, on the other hand, it is more commonly found in tubes of mud or fine sand in shallow water (Berkeley, 1927; Okuda, 1937; Monro, 1938; Hartman, 1961; Blake, 1966). Certainly the survival of the east coast shell-associated forms would be enhanced by larvae with a high degree of substrate selectivity and ability to delay metamorphosis.

TABLE III

Major differences between B. hamata populations on the east and west coasts of North America

East Coast	West Coast
Absence of transverse band of dark pigment on setiger 2 of larvae	Band present
Larval metamorphosis difficult to induce in the laboratory (apparent high substrate-specificity)	Larvae metamorphose readily on a variety of substrates in the laboratory
Metamorphosis can be delayed > 2 mo.; pelagic larvae up to 27 setigers obtained	Metamorphosis rapid; no pelagic larvae > 19 setigers; settle and form mucus tubes
Adults usually found in burrows in shells	Adults in a variety of substrates, algal holdfasts, and occasionally shells

Nothing is known about larval development and behavior of *B. hamata* populations from the Gulf of Mexico. Since many temperate Atlantic species had continuous distributions across northern Florida into the Gulf of Mexico during high sea levels in the Pleistocene (Hedgpeth, 1953), it is likely that larval development and behavior of Gulf coast populations are very similar to, if not identical with those of east coast populations.

SUMMARY

1. Larval development of the spionid polychaete, *Boccardia hamata,* is described from specimens followed from eggs and plankton isolates to young adult stages. Trochophore, 4-, 12-, 13- and 19-setiger stages are illustrated.

2. Eggs are deposited in an egg string within the *Boccardia* burrow. Development within the string proceeds to the 6-setiger stage at which stage the larvae are released to the plankton. Pelagic larvae grow to the 16- to 19-setiger stage before settling and metamorphosing. Length of pelagic life is estimated to be about three weeks.

3. Larval development of this species on both east and west coasts is identical except for slight variations in pigmentation and ventral ciliation on the third setiger. The most important difference between the larvae is in larval behavior at the time of metamorphosis. West coast larvae metamorphose readily on a variety of substrata and are unable to delay metamorphosis beyond the 19-setiger stage. East coast larvae are much more selective in choosing a substratum and can delay metamorphosis for more than two months. It is suggested that these behavioral differences reflect habitat differences of the adults on the two coasts.

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SODIUM, POTASSIUM, AND CHLORIDE TRANSPORT AND MEMBRANE POTENTIALS IN VALONIA VENTRICOSA

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In 1891 Meyer discovered that coenocytic cells of the marine alga *Valonia* have a high potassium and low sodium content. *Valonia* and other giant-celled algae subsequently became popular organisms for studies of ion permeability and transport, mainly because large quantities of vacuolar sap could be collected without contamination by protoplasm or extracellular fluid. From these early studies came Osterhout's theories on the nature of protoplasmic surfaces and his models of membrane transport by carrier molecules (Osterhout, 1931, 1940).

Electrical studies on *Valonia,* which were conducted with the permeability and transport studies, were initiated by Osterhout *et al.* (1924, 1927) and pursued later by Blinks (1940, 1955). *Valonia* was, in fact, the first cell in which a steady electric potential between internal and external solutions, *i.e.,* sap and sea water, was demonstrated. Osterhout's discovery that the vacuole of *Valonia* is electrically positive to external sea water did not seem unusual, since few data from other cells were available for comparison. The positive vacuole potential appears now, however, to be restricted to *Valonia* and its close relatives (see Blinks, 1949).

Later work on ion transport in *Valonia* suggested a relation between net K^+ uptake and external phosphate concentration (Mullins, 1949) and the existence of separate mechanisms for Na^+ and K^+ transport (Scott and Hayward, 1955). Another marine coenocyte, *Halicystis ovalis,* was shown to extrude Na^+ at the plasmalemma and actively absorb Cl^- at the tonoplast (Blount and Levedahl, 1960). Similar transports of Na^+ and Cl^- were found in *Nitellopsis,* a brackish-water characean (MacRobbie and Dainty, 1958a). Additional work on marine algae is summarized by Gutknecht (1965a).

In *Valonia* the relations between ion concentrations and fluxes and the electric potentials have not been studied. Therefore it is not known with certainty which ions are actively transported nor at which membrane, plasmalemma or tonoplast, the transport takes place. My objective is to answer these questions by using electrochemical methods similar to those of Dainty, MacRobbie and others working on the fresh-water Characeae (see reviews by Dainty, 1962, 1964).

I will show that the ionic and electrical properties of *Valonia ventricosa* differ in several respects from those of other plant cells. The high salt content of the vacuole is produced by an inward transport of Na^+ and especially K^+ at the tonoplast, whereas Cl^- uptake into the vacuole appears to be a passive process. The electric potential of the cytoplasm is highly negative to the vacuole, which contrasts with the small or zero potential across the tonoplast of other cells. Like most other plant and animal cells, *Valonia* actively extrudes Na^+ at the plasmalemma, and its cytoplasm is electrically negative to the external sea water.

METHODS

Culture methods

The *Valonia* cells used in this study were either 1–3 cm. diameter, shipped by air from Puerto Rico, or 0.3–0.8 cm. diameter, grown from aplanospores in the laboratory. Similar results were obtained with both sizes. The experimental and culture medium was an enriched sea water similar to that described by Keck (1964). Briefly, the medium consisted of natural sea water plus nitrate, phosphate, soil extract, and enough artificial sea salts (Utility Chemical Co., Paterson, N. J.) to raise the salinity to about 37‰—similar to the water from which the cells were collected.

Cultures were started with young cells, called aplanospores, which formed spontaneously from the protoplasm of mature coenocytes (see Steward, 1939). Soon after spore formation, the outer wall of the coenocyte was cleaned with alcohol to remove epiphytes. The wall was then pierced with a syringe, and a suspension of spores was withdrawn and transferred to the sea-water medium. Cultures started in this way were free from contaminating algae, which was important since other species usually grow faster than *Valonia*.

The cells were grown in large fingerbowls, and the medium was changed at about two-week intervals. The temperature was 25 ± 1° C., and illumination was 3000–4000 lux, 8–12 hr./day (natural daylight plus fluorescent lighting). Some of the bowls contained marble chips, as prescribed by Steward (1939); within several weeks many cells became attached to the marble substrate by rhizoid processes. This attachment did not appear necessary for normal growth and development, however, for in three months both attached and unattached cells grew to a diameter of about 0.5 cm. and showed similar ionic and electrical properties.

Chemical measurements

Sap from large cells was obtained by piercing the cell surface with a syringe and withdrawing about 200 μl. Sap from small cells was collected by washing the cell for one minute in 0.9 *M* sucrose, blotting with absorbent tissue, puncturing with a sharp needle, and gently squeezing. The sap was uncontaminated by protoplasm, as indicated by the absence of chloroplasts in the sample.

Protoplasm was collected by cutting a large coenocyte in half and allowing the sap to drain out. The wall and adhering protoplasm were washed gently for 20–25 seconds in a large volume of Tris-sulfate buffer, pH 7.7–7.8, approximately isosmotic to sap (1.14 molal). The protoplasm, along with some buffer, was gently scraped from the wall with a scalpel, taken up in a capillary tube, and centrifuged in a hematocrit centrifuge for 10 minutes at about 12,000 *g*. The protoplasm fraction was forced out of the tube and weighed. The protoplasm from several large cells was pooled to give 20–40 mg. wet weight per sample. The protoplasm was combined with the supernatant buffer, diluted to 5 ml. with 0.1 N HNO_3, and allowed to extract for several days.

The amount of water in the protoplasm was measured in separate samples by drying overnight at 100–110° C. Na^+, K^+, and Cl^- were measured with a Beckman DU flame spectrophotometer and an Aminco-Cotlove chloride titrator. The osmotic pressure of sea water, sap, and Tris was measured with a Fiske osmometer.

This method of collecting protoplasm was not quantitative, for there was always some loss during washing and scraping. The amounts agreed approximately, however, with those predicted from Doyle's (1940) measurements of protoplasm volume and density. No attempt was made to separate the cytoplasm from the chloroplasts, which comprise 60–70% of the protoplasm volume in *V. ventricosa* (Doyle, 1935, 1940).

Electrical measurements

Electric potentials of the vacuole and cytoplasm were measured with microcapillary electrodes and a high-impedance voltmeter (Keithley Model 600A). The electrical circuit was similar to that described by Gutknecht (1965a). Micropipets were drawn from 1-mm. Pyrex tubing and filled with 0.6 M KCl by boiling under reduced pressure. Tip diameters ranged from <1 μ to about 10 μ. The larger tips were necessary to pierce the tough outer wall of old cells. Electrode tip potentials in either sea water or artificial sap were <2 mv.

To measure the vacuole potential, a microelectrode was forced through the cell wall and underlying protoplasm (5–10 μ thick) until the tip protruded about 1 mm. into the vacuole. The puncture was made with the aid of a micromanipulator and dissecting microscope. The potential difference between vacuole and sea water was initially low, then rose gradually to a maximum and remained steady (± 2 mv) for many hours. The cells were illuminated during both the electrical and flux measurements (3000–4000 lux), which was important because the vacuole potential in *V. ventricosa* is sensitive to light (Marsh, 1939).

The electric potential of the cytoplasm was measured in aplanospores 5–15 hours old and 100–300 μ in diameter. These young cells were largely protoplasm rather than vacuole, and their thin wall offered little resistance to microelectrode penetration. The cells were held by gentle suction, similar to the method of Eckert (1965). When the electrode tip entered the cytoplasm, the potential rose rapidly to a maximum, then usually decreased slowly after 1–2 minutes. This decrease was due possibly to a cytoplasmic sealing process like that in *Nitella* (Walker, 1955). Potentials that were steady (± 2 mv) for 1–2 minutes were recorded.

Aplanospores of fairly uniform age were obtained by cutting a large cell in half and holding it in sea water for several hours. During this time the protoplasm changed from a continuous layer into a large number of multinucleate spores, 20–300 μ in diameter (see Doyle, 1940). Alternatively, a small coenocyte was centrifuged at 1000 g for 10 minutes and the protoplasm, concentrated at one end of the cell, was allowed to stand for several hours in sea water. Spores formed by the latter method were usually larger but gave similar results.

Flux measurements

Ion fluxes between vacuole and sea water were measured in spherical cells of *Valonia*, 5–6 mm. in diameter and 60–110 mg. in weight. The small rhizoidal cells and hapteron cells on the surface of the main vesicle were removed with forceps. Unidirectional fluxes were measured with radioisotopes, Na^{22}, K^{42}, and Cl^{36}. Conventional detectors were used (Baird-Atomic Model 510 and Nuclear Chicago Model C-115). Tracer specific activities were high enough that the concentrations of stable ions in the radioactive media were increased less than 1%. Net fluxes were measured by the weight increase of a cell during an influx experiment (1–3 days). This was possible because the intracellular ion concentrations were constant and the average rate of growth was about 1 mg./day. Efflux was calculated from the unidirectional influx minus the net influx. Efflux of Na^+ and K^+ was also measured directly from the rate of isotope efflux into a large non-labeled environment. Both methods gave similar results, but the former method was preferred because it provided simultaneous estimates of both influx and efflux in a single cell.

To measure the influx from sea water to vacuole, a weighed cell was placed in 10 ml. of labeled medium under constant illumination. Cells were removed after 20–50 hours, washed for one minute in non-active medium, blotted, weighed, measured, and pierced with a sharp needle. The sap was squeezed out, taken up in a calibrated pipet, and transferred to a planchet for counting. The initial influx was calculated by the equation

$$M_i = \frac{C_i^* C_o V}{t C_o^* A}$$

where M_i is the influx in M/cm^2. sec., C_i^* is the concentration of labeled ions in the sap after time t, C_o is the external ion concentration, C_o^* is the external concentration of labeled ions, V is the volume, and A is the surface area of the spherical cells. The specific activity of the sap at the end of the influx period was less than 10% of the specific activity outside; therefore the efflux of labeled ions during the influx measurement was neglected.

The net flux was calculated by the equation

$$M_{net} = \frac{\Delta W C_i}{At}$$

where M_{net} is the net influx in M/cm^2. sec., ΔW is the weight increase during time t, and C_i is the internal ion concentration.

Efflux was estimated either by subtracting the net flux from the unidirectional influx, or from direct measurements of the rate of isotope efflux into a large non-labeled environment by the equation

$$M_o = \frac{2.3 C_i V}{tA} \log \frac{C_{i0,}^*}{C_i^*}$$

where M_o is the efflux in M/cm^2. sec., and $C_{i,0}^*$ is the initial concentration of labeled ions inside.

These equations were derived from standard flux equations which treat the cells as a single compartment (see, for example, Sheppard, 1962). This was justified because the cells used for flux measurements were $>99\%$ vacuole.

RESULTS

Ion concentrations in sea water, protoplasm, and sap

Concentrations of Na^+, K^+, and Cl^- in sea water, protoplasm, and vacuolar sap are shown in Table I. Sap was analyzed from large Puerto Rican cells and from smaller cells grown in the laboratory. Protoplasm was collected only from the large coenocytes, since the small cells contained only about 0.5 μl. protoplasm per cell. Protoplasmic ion concentrations are expressed in terms of protoplasm water, which was 58.6 ± 1.0% (10) of the wet weight. Results are presented in the sequence: mean, standard error, and (in parentheses) number of measurements.

Potassium was highly concentrated in the protoplasm (434 m*M*) and especially in the vacuole (625 m*M*). Sodium was largely excluded from both protoplasm (40 m*M*) and sap (44 m*M*). The Cl^- concentration was relatively low in the protoplasm (138 m*M*), but was higher in the sap (643 m*M*) than in the external sea water. These concentrations for the sap of *V. ventricosa* are slightly higher than those of Cooper and Blinks (1928) and are similar to those of Steward and Martin (1937).

Table I

*Concentrations of Na^+, K^+, and Cl^- in sea water and protoplasm and sap of Valonia ventricosa**

Ion	Sea water (m*M*/liter)	Protoplasm (m*M*/liter water)	Sap (m*M*/liter)
Na^+	508 ± 5 (13)	40 ± 2 (8)	44 ± 1 (18)
K^+	12.1 ± 0.2 (10)	434 ± 9 (8)	625 ± 5 (18)
Cl^-	596 ± 2 (9)	138 ± 4 (8)	643 ± 2 (10)

* Mean, SE, and number of measurements.

Electric potentials across plasmalemma and tonoplast

The potential difference between cytoplasm and sea water was −71 ± 1 mv (20). The measurements were made on cells 5–10 hours old, which were mainly protoplasm surrounded by a thin cell wall. Vacuoles in cells of this age were small and not clearly defined (see Doyle, 1935). When the electrode was forced toward the center of these cells, the potential sometimes reversed sharply from negative to positive (+6 to +14 mv), which suggested entry into a vacuole. Cells 20–30 hours old resembled mature *Valonia* in having a large vacuole surrounded by a thin layer of protoplasm. In cells of this age I was sometimes able to measure a cytoplasmic potential of −70 ± 5 mv (6), whereas the vacuole potential was +12 ± 1 mv (12).

The potential difference between vacuole and sea water in older cells (3–4 months) was +17 ± 1 mv (16), vacuole positive. These cells were illuminated continuously for 2–3 days prior to the potential measurements to simulate conditions under which ion fluxes were measured. This value agrees with the early work of Blinks (1929), who used much larger, and presumably much older, cells of *V. ventricosa*.

The vacuole potential is the algebraic sum of the potentials across plasmalemma and tonoplast. Thus, subtracting the plasmalemma potential in young cells (−71 mv) from the vacuole potential in mature cells (+17 mv) gives a tonoplast potential of about −88 mv, cytoplasm negative to vacuole. This value should be confirmed by direct measurements on large cells. I have not accomplished this, however, mainly because the cell wall is as thick as the underlying protoplasm (5–12 μ; Doyle, 1940) and cannot be penetrated easily by a glass capillary of 1 μ diameter.

These results support qualitatively the early work of Umrath (1938), who found that the potential difference between cytoplasm and sea water in *V. macrophysa* was about −20 mv. Umrath measured cytoplasmic potentials in slowly deplasmolyzing cells by allowing the protoplasm to be pierced by a stationary microelectrode inside the cell wall. His microelectrodes were filled with a too dilute solution of KCl (0.1 *M*), however, for which he was criticized by Briggs, Hope and Robertson (1961, p. 109). So, although his method was not entirely satisfactory, he concluded correctly that the cytoplasm of *Valonia* is more negative toward the vacuole than toward the external sea water.

Ion fluxes

Ion fluxes between sea water and vacuole of *V. ventricosa* are shown in Table II. Unidirectional fluxes ($\mu\mu M/\text{cm}^2$. sec.) were 3.3–3.6 for Na^+, 86–89 for K^+, and 11–18 for Cl^-. Influxes were somewhat larger than effluxes, because the cells were growing at a rate of 0.5–1.5%/day and the intracellular concentrations were constant. The net fluxes, estimated from the increase in weight and average concentration of ions in the sap, were about 0.3 for Na^+, 5.6 for K^+, and 7.6 for Cl^-.

TABLE II

Fluxes of Na^+, K^+, and Cl^- between sea water and vacuole *, **

Ion	Influx	Efflux	Net flux
Na^+	3.64 ± 0.32 (20)	3.32 ± 0.33 (20)	0.32 ± 0.03 (35)
K^+	88.7 ± 3.0 (18)	86.2 ± 3.0 (18)	5.6 ± 0.6 (33)
Cl^-	18.5 ± 1.4 (30)	10.9 ± 1.1 (30)	7.6 ± 0.5 (30)

* Mean, SE, and number of measurements.
** Units are $\mu\mu M/\text{cm}^2$. sec.

The efflux of Na^+ and K^+ from *V. ventricosa* was also measured directly from the rates of Na^{22} and K^{42} efflux into a large non-labeled environment. The cells were labeled for only a short time (1–5 hours), so that the specific activity of the protoplasm would be higher than that of the sap at the start of the efflux measurement. Some typical results are shown in Figure 1. A rapid loss of both Na^{22} and K^{42} from the wall and extracellular space was complete in about 0.5 minute. The remaining Na^{22} exchanged at a nearly uniform rate and probably represents exchange of vacuolar Na^+. The rate of K^{42} exchange was non-uniform during the first hour but was constant thereafter, presumably representing exchange of vacuolar K^+. Effluxes of Na^+ and K^+ from vacuole to sea water were estimated from the slopes of the linear parts of the curves, K^{42} B and Na^{22}, as described under Methods. The efflux of Na^+, measured between 0.3 and 65 hours, was 3.8 ± 0.3 (8) $\mu\mu M/\text{cm}^2$. sec. The efflux of K^+, measured between 1 and 26 hours, was 89 ± 9 (5) $\mu\mu M/\text{cm}^2$. sec. These values agree with those obtained by the more convenient method of subtracting the net influx from the unidirectional influx (Table II).

Preliminary calculations suggest that the exchange of intracellular K^{42} proceeds at two exponential rates. The rapidly exchanging fraction (K^{42} A) was obtained by subtracting the extrapolated portion of the slow fraction (K^{42} B) from the total activity. The faster rate probably represents exchange of protoplasmic K^+ rather than cell-wall K^+, based on the rate of exchange and the fact that an analogous compartment for Na^+ was not observed. The fast fraction had an apparent half-time of about 0.5 hour and a rate coefficient of about 1.3 hr.$^{-1}$. Efflux of K^+ from protoplasm to sea water was estimated by multiplying the rate coefficient by the K^+

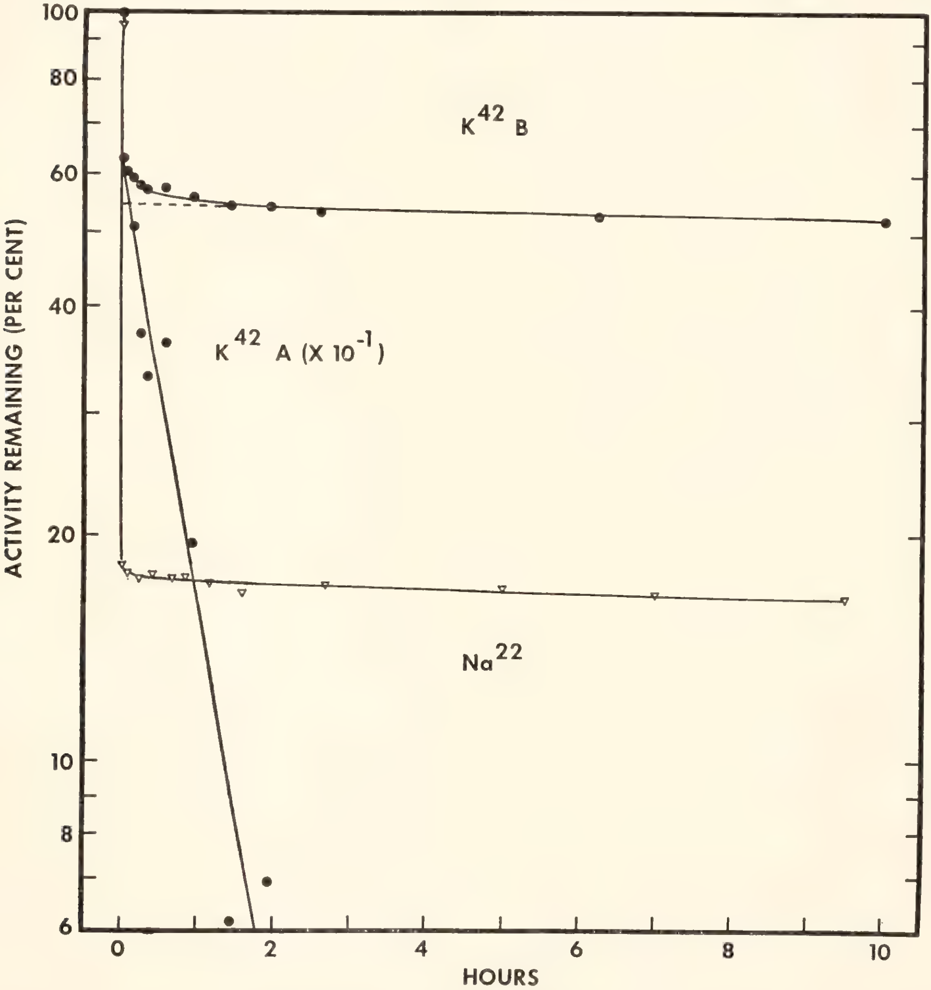

FIGURE 1. Efflux of Na^{22} and K^{42} from cells of *V. ventricosa*, previously labeled for 5 hours (Na^{22}) or one hour (K^{42}) in the light. Rapidly exchanging fraction of intracellular K^{42} (K^{42} A) was obtained by subtracting the extrapolated portion of the slow fraction (K^{42} B) from the total activity. Each point represents a measurement of 11–30 cells.

concentration of the protoplasm (about 13×10^{-8} M/cm^2.), which gave a value of about 50 $\mu\mu M/\text{cm}^2$. sec.

The K^+ flux from protoplasm to sea water, the rate coefficient, and the apparent size of the protoplasm compartment are all underestimated, however, by the graphical analysis of the washing-out curve (Fig. 1). The error is caused by the series arrangement of protoplasm and vacuole, the relatively small size of the protoplasm compartment, and the similarity between the fluxes across plasmalemma and tonoplast (see Solomon, 1960; Huxley, 1960). First, the protoplasm loses radioactivity to both vacuole and sea water during the washing-out process. Second, the specific activity of the protoplasm after several hours in a K^{42} medium is probably not equal to the specific activity of the medium, but is at a quasi-steady level below that of the medium.

Discussion

The occurrence and direction of any active transport processes in *V. ventricosa* can be determined from a knowledge of the ion concentrations and electric potentials under steady-state conditions. Active transport is indicated by any large differences between the electrochemical potentials of an ion in sea water, cytoplasm, or sap. The difference in electrochemical potential can be estimated by comparing the measured membrane potential with the Nernst equilibrium potential, *i.e.,* the potential at which the ion would be in passive flux equilibrium between the two phases.

$$E_j = \frac{2.3RT}{zF} \log \frac{C_1}{C_2}$$

E_j is the equilibrium potential, C_1 and C_2 are the ion concentrations on either side of the membrane, z is the algebraic valency, and R, T, and F have their usual meanings. In the discussion which follows, the sign of the potential will always be that of the cytoplasm. The sea water and vacuole are taken as ground for the potentials across the plasmalemma and tonoplast, respectively.

Possible active transports of Na^+ and K^+ in *V. ventricosa* may be tested by using the Nernst equation, because these ions are in an approximately steady-state (Table II). Equilibrium potentials for Na^+ and K^+ across plasmalemma and tonoplast are shown in Table III. Also shown are the differences, ΔE, between the measured potential and the equilibrium potential for each ion. The magnitude of the ΔE indicates the relative driving force acting on the ion. The sign of the ΔE, along with the valency of the ion, indicates the direction of that driving force. The large values for ΔE_{Na} at the plasmalemma (−136 mv), and for both ΔE_{Na} and ΔE_K at the tonoplast (−90 and −97 mv) indicate active transport of these ions. An active efflux of Na^+ from protoplasm to sea water and active transports of both Na^+ and K^+ from protoplasm to vacuole are indicated. An active influx of K^+ at the plasmalemma is likely, although the ΔE_K at this membrane is relatively small (+21 mv).

Where marked deviations from flux equilibrium occur, as with Cl^- (Table II), the flux-ratio equation of Ussing (1949) and Teorell (1949) may be used to test

TABLE III

*Equilibrium potentials for Na^+ and K^+ across plasmalemma and tonoplast**

Ion	Plasmalemma		Tonoplast	
	E_j**	$\Delta E = E_{obs} - E_j$	E_j**	$\Delta E = E_{obs} - E_j$
Na^+	+65	−136	+2	−90
K^+	−92	+21	+9	−97

* Calculated from data in Table I.

** $E_j = \frac{-2.3RT}{zF} \log \frac{C_i}{C_o}$ millivolts.

for passive, independent, ion movement.

$$\frac{M_i}{M_o} = \frac{C_o}{C_i \exp(zFE/RT)}$$

M_i/M_o is the predicted flux ratio (influx/efflux), E is the measured vacuole potential, and the other symbols have the same meanings as before. Close agreement between predicted and measured flux ratios is strong evidence for passive ion movement. Discrepancies suggest, but do not prove, active transport, since such passive processes as exchange diffusion and single-file diffusion can cause deviations from the predicted flux ratios (see Ussing, 1960, p. 49).

Measured and predicted flux ratios for Na^+, K^+, and Cl^- between sea water and vacuole are shown in Table IV. The predicted values were calculated from the average vacuole potential of +17 mv and the sap and sea-water concentrations shown in Table I. The large differences between the measured and predicted values for both Na^+ and K^+ support the earlier indications of active efflux of Na^+ and active influx of K^+. On the other hand, the close agreement between the predicted and measured flux ratios for Cl^- (1.70 and 1.79) indicates that Cl^- uptake into the vacuole of growing cells is a passive process.

The absence of active transport of Cl^- from sea water to vacuole, however, does

TABLE IV

Measured and predicted flux ratios for Na^+, K^+, and Cl^- between sea water and vacuole

Ion	Influx/Efflux, M_i/M_o	
	Measured*	Predicted**
Na^+	1.10	5.78
K^+	1.03	0.01
Cl^-	1.70	1.79

* Calculated from data in Table II.

** $\frac{M_i}{M_o} = \frac{C_o}{C_i \exp(zFE/RT)}$.

not preclude the possibility of some active uptake of Cl^- into the protoplasm at both membranes. The predicted flux ratio at the plasmalemma (M_{oc}/M_{co}) is about 0.3, and the predicted flux ratio at the tonoplast (M_{cv}/M_{vc}) is about 6. The actual flux ratios are not known, but the measured value of 1.7 between sea water and vacuole (M_{ov}/M_{vo}) sets an upper limit on the flux ratios. A lower limit of about 1 is set by the experimental conditions, *i.e,* growing cells which maintain a constant Cl^- concentration inside. Thus, the actual flux ratios at plasmalemma and tonoplast must be 4–6 times higher and lower, respectively, than the predicted flux ratios. This suggests either that there is active uptake of Cl^- into the protoplasm at both membranes or that the estimated Cl^- concentration in the protoplasm is too high. The latter possibility is more likely, because either contamination by sap or the presence of minute sap-filled vesicles in the protoplasm (see Buvat, 1963; Sutcliffe, 1962) could raise the estimated Cl^- concentrations above the equilibrium level. Two other possible sources of error are the ion selectivity of the chloroplasts and any changes in the cytoplasmic potential with age.

Valonia ventricosa differs from other plant cells by not showing active uptake of Cl^- into the vacuole. Active Cl^- absorption occurs, for example, in the marine algae *Rhodymenia, Halicystis,* and *Gracilaria* (MacRobbie and Dainty, 1958b; Blount and Levedahl, 1960; Gutknecht, 1965a). In giant characean cells, as well as in roots, storage tissues, and leaves of higher plants, an inward Cl^- or anion pump is essential for maintaining the high salt content of the vacuole (see Dainty, 1964). In *V. ventricosa,* however, Cl^- appears to follow passively while Na^+, and especially K^+, are pumped into the vacuole. Thus, the hypertonicity of the cell interior, which is essential for the growth and expansion of all walled cells (see Rothstein, 1964), is primarily due to an inward transport of cations rather than anions. In this respect *Valonia* resembles yeast and bacteria more than other algae and higher plants.

Active transport of one or more ions from cytoplasm to vacuole is necessary for osmotic reasons, summarized recently by Tosteson (1964). Otherwise, unless the tonoplast were pressure-resistant, the protoplasm would be osmotically unstable toward the sap. For this and other reasons the inward Cl^- pump in both *Halicystis* and *Nitellopsis* was sited at the tonoplast (Blount and Levedahl, 1960; MacRobbie and Dainty, 1958a). In *Nitella* it appears that Na^+ is pumped in at the tonoplast, whereas K^+ and Cl^- are in equilibrium between cytoplasm and sap (Spanswick and Williams, 1964). In *V. ventricosa,* however, it is primarily K^+ which is pumped from cytoplasm to sap.

The energy requirement for K^+ transport in *V. ventricosa* is of interest because of the rapid influx of K^+ (89 $\mu\mu M/cm^2$. sec.) and the large electrochemical potential gradient against which K^+ is pumped (about 2500 cal./M). The cells in which K^+ fluxes were measured contained about 5×10^{-4} g. protoplasm cm². The energy requirement is thus about 1.6 cal./g. protoplasm per hour, which is rather high and suggests that part of the K^+ fluxes may be due to a mediated, but not active, process, such as exchange diffusion (see Ussing, 1960).

The large potential across the tonoplast is another unusual feature of *V. ventricosa.* The marine alga *Halicystis ovalis* and root hair cells of *Avena* show no potential across the tonoplast (Blount and Levedahl, 1960; Etherton and Higinbotham, 1960). Fresh-water Characeae show either no potential or very small potential (Walker, 1955; Kishimoto, 1959; Nagai and Kishimoto, 1964; Findlay and Hope, 1964; Spanswick and Williams, 1964).

At the plasmalemma, both the large negative potential and the active extrusion of Na^+ are features common to many cells. Two other marine algae, *Halicystis* and *Porphyra*, show cytoplasmic potentials of −80 and −42 mv, respectively (Blount and Levedahl, 1960; R. W. Eppley, personal communication). In fresh-water algae and higher plants the plasmalemma potentials are usually somewhat larger (Walker, 1955; Umrath, 1956; Findlay and Hope, 1964; Spanswick and Williams, 1964). Finally, the outward transport of Na^+, possibly coupled with an inward transport of K^+, occurs at the outer plasma membrane of many plant and animal cells (see Dainty, 1962, 1964).

The suitability of *Valonia* for salt-transport studies is questioned by Steward

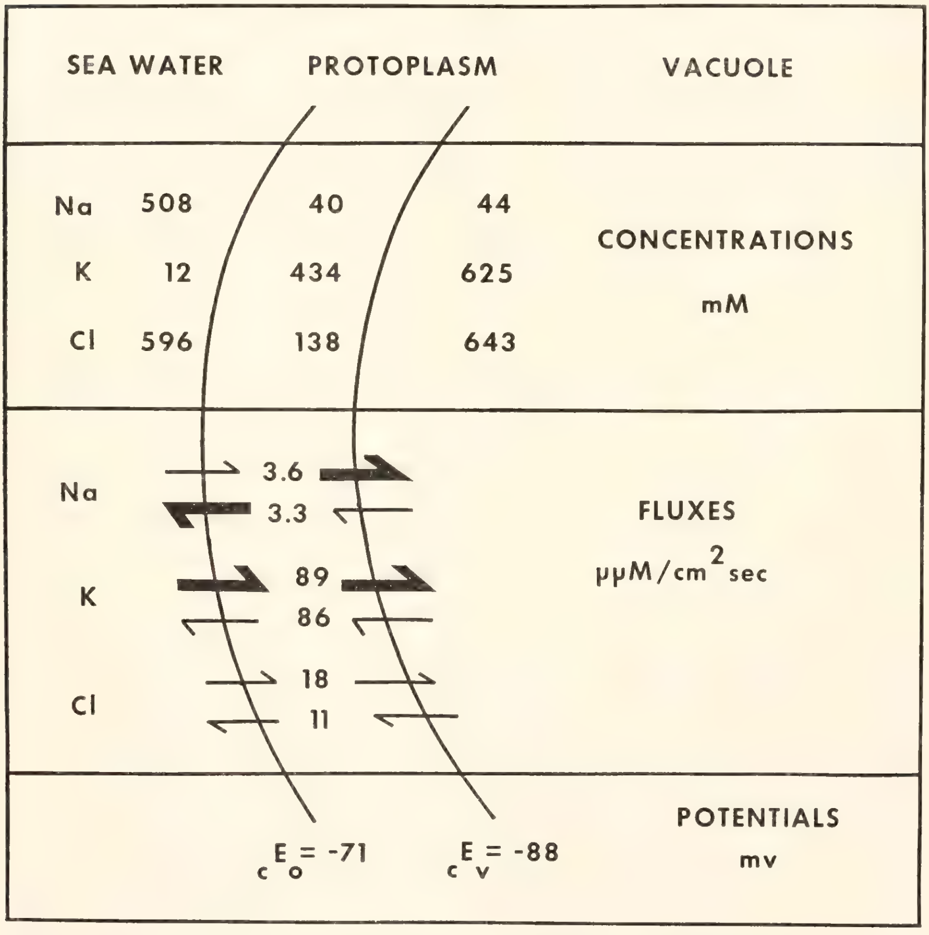
SEA WATER
PROTOPLASM
VACUOLE
Na 508 40 44
K 12 434 625
Cl 596 138 643
CONCENTRATIONS
mM
Na 3.6 3.3
K 89 86
Cl 18 11
FLUXES
μμM/cm² sec
POTENTIALS
mv
$_cE_o$ = -71
$_cE_v$ = -88

FIGURE 2. Ionic relations in *V. ventricosa*. Provisional scheme showing concentrations, fluxes, and potentials. Large arrows indicate active transport.

and Sutcliffe (1959, p. 289), because the sap is not subject to rapid changes in composition, and because the cells grow slowly and only from aplanospores which send rhizoidal processes into the substratum, *e.g.*, marble chips. They suggest that the rhizoidal processes, as well as the small hapteron cells on the surface of the main vesicle, may be the functional absorbing organs. My results indicate that neither the rhizoidal cells, nor the substratum, nor the hapteron cells are necessary for fairly rapid growth and rapid ion movements. The growth rate of 0.5–1.5% per day in fairly large cells (60–110 mg.) is comparable with that in some other marine algae under fairly similar conditions (Gutknecht, 1965b), and the fluxes of ions across the surface of the main vesicle are more rapid than in most other plant and animal cells. That the sap is not subject to rapid changes in composition is probably due mainly to the large volume:surface ratio.

A provisional scheme of the ionic relations in *V. ventricosa* is shown in Figure 2. The important differences between *V. ventricosa* and other vacuolated plant cells are (1) the absence of active transport of Cl^- into the vacuole, (2) the inward transport of both Na^+ and K^+ at the tonoplast, and (3) the large potential across the tonoplast. Two features of *V. ventricosa* that are similar to other vacuolated plant cells, as well as to plant and animal cells in general, are (1) the active efflux of Na^+ and probably active influx of K^+ at the plasmalemma, and (2) the large negative potential of the cytoplasm relative to the external solution. Thus, the main differences between *Valonia* and other plant cells lie at the vacuole membrane. These results may therefore support the view of Steward (1939) and Steward and Sutcliffe (1959), based on morphological evidence, that the vacuole of *Valonia* and its relatives is not homologous to the vacuole of most other plant cells.

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SUMMARY

1. The marine alga *Valonia ventricosa* regulates its ionic content by means of several ion pumps. An active extrusion of Na^+ and probably an active uptake of K^+ occur at the plasmalemma. At the tonoplast there is an inward transport of Na^+ and especially K^+. The evidence for these ion pumps is that Na^+ efflux at the plasmalemma and Na^+ and K^+ influx at the tonoplast occur against large electrochemical gradients under steady-state conditions.

2. Ion distribution and membrane potentials across plasmalemma and tonoplast were measured. The ion concentrations were: Na^+ 508, K^+ 12.1, and Cl^- 596 mM/liter sea water; Na^+ 40, K^+ 434, Cl^- 138 mM/liter protoplasm water; Na^+ 44, K^+ 625, Cl^- 643 mM/liter sap. The resting potential across the plasmalemma was about 71 mv, cytoplasm negative to sea water. The potential across the tonoplast was about 88 mv, cytoplasm negative to sap. The large potential across the tonoplast is a feature not found in other vacuolated plant cells.

3. Ion fluxes between sea water and vacuole were measured in growing cells of *Valonia*. The fluxes were: Na^+ 3.3–3.6, K^+ 86–89, Cl^- 11–18 $\mu\mu M/cm^2$. sec. The ratio of influx:efflux for Na^+ and K^+ was close to unity, whereas the flux ratio for Cl^- was 1.70. The latter value is close to that predicted by the Ussing-Teorell equation for a passively moving ion, which suggests that *Valonia*, unlike other vacuolated plant cells, does not actively transport Cl^- into the vacuole.

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REPRODUCTIVE AND MOLTING CYCLES IN CAVE CRAYFISH

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In the epigean environment, crayfish of temperate North America are subjected to annual fluctuations of many factors including organic material, various dissolved chemicals in the water, temperature and length of photoperiod. The latter two exhibit striking changes, rising in the spring from winter minima to summer maxima and lowering in the fall to the winter minima. The effects of changing temperature and photoperiod on reproductive cycles have been documented for many vertebrate and invertebrate animals.

For the majority of North American crayfishes that have been studied the pattern of annual reproduction is for breeding to occur predominantly during the fall, while egg-laying occurs during the spring after temperatures begin to rise and photoperiods are increasing. This pattern has been reported for *Orconectes obscurus* and *Cambarus diogenes* (Ortmann, 1906), *O. limosus* (Ortmann, 1906; Crocker, 1917), *O. propinquus* [= *Cambarus propinquus*] (Van Deventer, 1937; Bovbjerg, 1952; Crocker, 1957) and *C. longulus* (Smart, 1962). An annual cycle of spermatogenesis in *C. montanus* (Word and Hobbs, 1958) and of ovarian development in *C. longulus* (Smart, 1962) has been demonstrated. Molting of adult crayfish usually occurs twice a year, in late spring (spring molting period) and early fall (summer or fall molting period). Such a pattern of molting has been reported for *O. immunis* [= *Cambarus immunis*] (Tack, 1941), *O. limosus* and *O. obscurus* (Ortmann, 1906), *O. propinquus* [= *Cambarus propinquus*] (Van Deventer, 1937; Crocker, 1957), *C. longulus* (Smart, 1962) and *C. montanus* (Word and Hobbs, 1958).

Experiments by various investigators (Word and Hobbs, 1958; Lowe, 1961; Stephens, 1952; Stephens, 1955) have shown that light and temperature do affect reproductive and molting cycles of crayfish. Therefore, it is of considerable interest to examine reproductive and molting activities in cave crayfish living in a subterranean environment where there is no light and where yearly temperature changes may be small.

Materials and Methods

The data presented in this paper were collected during the years 1960–65 from the population of cave crayfish, *Orconectes pellucidus inermis,* living in Shiloh Cave near Bedford, Indiana; studies were conducted in the cave during several months of each year. These crayfish frequent the pools and slow-moving areas of the meandering stream that flows through the cave. The size of the population was measured by the Petersen proportional method (Dice, 1952). It is small in Shiloh Cave compared to the size of crayfish populations observed in epigean streams, and was estimated to be about 200 large juveniles and adults during the fall of 1962.

The number of crayfish sampled on any one date varied from 7 to 100 for the 26 trips to the cave during this study. The amount of stream habitat available for study is over 1100 meters in length with an average of 1.4 meters in width. Depth normally varies from 0.1 to 1.0 meter but may increase considerably after flooding periods. The water is clear during normal flow but increases in turbidity during flooding periods, due to silt suspension and influx of organic material. Annual records of water temperature varied by only 1° C.; the low of 12.2° C. was recorded in December and the high of 13.2° C. in August.

The reproductive cycle of females was studied by observing when females carry eggs, when newly hatched crayfish are present in the stream, and when changes in the ovary and cement glands occur (cement glands secrete material for gluing the eggs to the abdominal appendages, the pleopods). The ovary can be observed through the translucent carapace. Changes in the testes cannot be observed through the carapace; thus, the reproductive cycle of males was studied from histological sections. Testes were fixed in alcohol-formalin-acetic acid (AFA) mixture during several months of the year and, after the usual histological procedures, stained by either Mallory's triple connective tissue procedure (Guyer, 1953) or Delafield's hematoxylin and eosin. In addition the change in ratio of breeding to non-breeding adult males (differences in these males are discussed below), which is an important means for evaluation of periodic molting and reproductive activities, was compiled on each trip to the cave.

The molt cycle was categorized according to the classification of Carlisle and Knowles (1959) and Carlisle (1960): premolt, ecdysis, postmolt and intermolt. Several criteria were used to stage crayfish in the molt cycle including (1) occurrence of ecdysis, (2) relative hardness of the exoskeleton and (3) relative darkness of the exoskeleton. Animals shedding the exoskeleton are rarely seen because ecdysis occurs within an hour; however, the individual has a very soft exoskeleton for about a day before and after ecdysis. It is flexible when touched for a few weeks before ecdysis and for a week or more after ecdysis. This flexibility occurs before molting when calcium is being removed from the old shell and after molting before calcium deposition in the new shell is completed. Body pigments are lacking in *O. p. inermis;* the exoskeleton is white after ecdysis, but darkens with time due to the deposition of materials suspended in the water and perhaps to changes within the exoskeleton itself. In addition to these criteria crayfish were marked with fingernail polish during the fall months as an aid in clarifying a fall molting period.

Female Reproductive Cycle

Information about the reproductive cycle of cave crayfish is sparsely recorded in the literature. Park, Roberts and Harris (1941) reported the occurrence of one ovigerous female (carrying eggs on the abdominal appendages) on June 26 and two during the middle of September. According to them a breeding periodicity in *O. p. pellucidus* appears to be poorly developed. Giovannoli (1933) reported that ovigerous females are found during the winter; from this observation Brown (1961) assumed that an annual reproductive rhythm was present in this cave crayfish. However, Giovannoli's information was taken from Hay (1903, p. 232): "Eggs are said to be laid during the winter, but the guides were rather indefinite

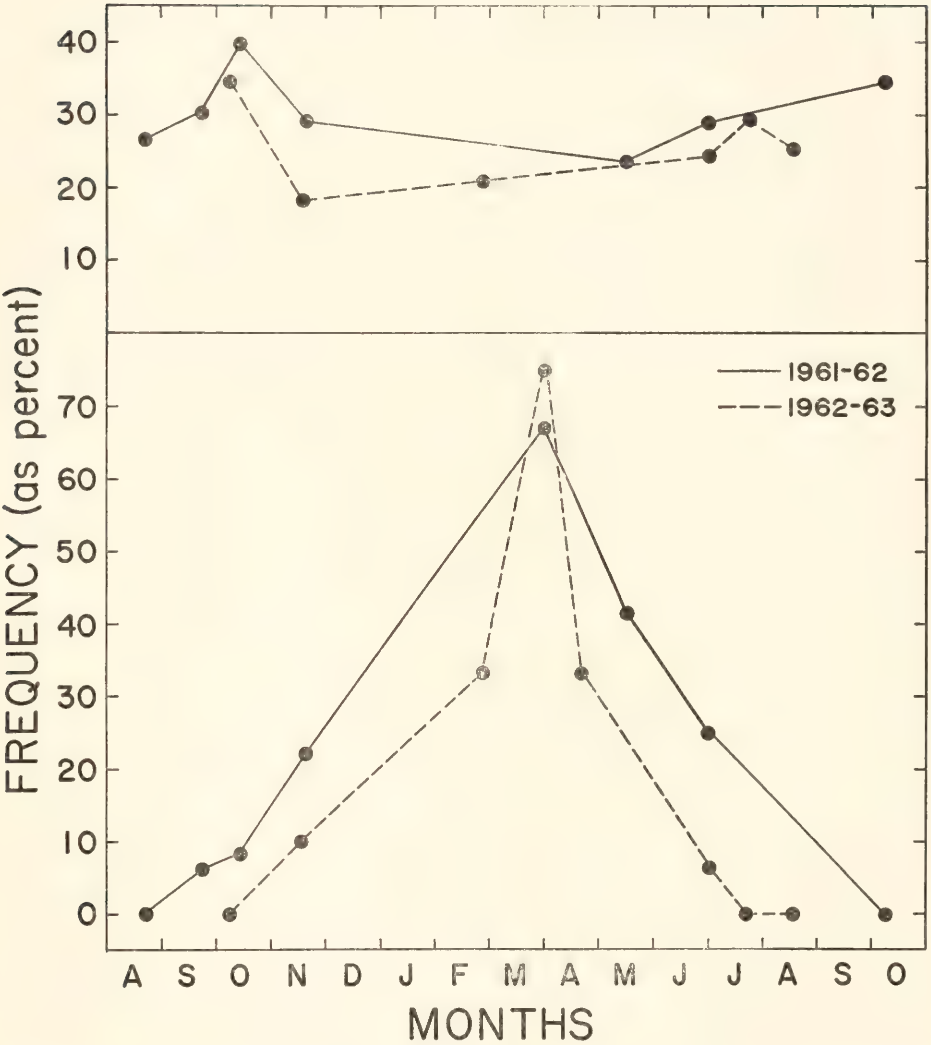

FIGURE 1. The frequency of adult females having large, yolky oocytes in the ovary, stage 4, (bottom) and the frequency of immature crayfish <18 mm. carapace length (top) in the population of *O. p. inermis* in Shiloh Cave. The number of adult females and of immature animals varied, respectively, from 3 to 24 and from 12 to 37 in samples taken during the two years.

as to the exact time." Poulson (1964) suggested that *O. pellucidus* may have a fall breeding period.

OBSERVATIONS ON FEMALE REPRODUCTIVE CYCLE

Five ovigerous females were found in Shiloh Cave, all from June 30 to August 21. An ovigerous female collected on August 16, 1963, had the most advanced embryos, and these embryos required less time to develop in the laboratory at 15–17° C. than embryos from the other females. Upon hatching the young crayfish remain attached to the adult until after the second molt; they are less than 5 mm. in carapace length after leaving the adult and entering the stream population. A few small crayfish (<12 mm. carapace length) were found during all of the months except January, March and April, but they are most numerous and conspicuous during the summer and fall months; the largest number was recorded during August, 1964.

A good indicator of an annual cycle of reproduction in a crayfish is the cycle of oocyte formation and growth in the ovary. The ovary in crayfish is a trefoil organ situated between the floor of the pericardial sinus and alimentary canal. In *O. p. inermis,* the quiescent ovary lies in a plane nearly parallel to the dorsal and ventral surfaces of the carapace. But the anterior lobes grow upwards as the ovary increases in volume, nearly reaching the dorsal carapace in the advanced stages of development. The following stages in ovarian development of adult and of large immature crayfish have been used in this study: (1) oocytes are small (<0.3 mm. in diameter), white in color, and barely visible without magnification; (2) oocytes are somewhat larger (largest oocytes 0.3–0.6 mm. in diameter), white in color, and the anterior lobes of the ovary are turned upwards, due to an increased number of oocytes; (3) oocytes are large (largest oocytes 0.6–1.3 mm. in diameter), white to yellow in color, and the anterior lobes nearly reach the dorsal carapace; and (4) oocytes are very large (largest oocytes > 1.3 mm. in diameter) and yellow to orange in color.

Data of the percentage of females with large, yolky oocytes (stage 4) were obtained during two consecutive years, beginning during the fall of 1961. The number of adult females sampled varied from 3 to 24. The data clearly show an annual cycle for each of the two years sampled (Fig. 1). The lowest frequency of females with large, yolky oocytes in their ovaries occurs during the late summer and early fall months. The frequency rises during late fall and winter months, reaches a peak during early spring months and declines during late spring and summer months as eggs are laid by some females and perhaps as oocytes are resorbed in others.

A new annual period of oocyte formation begins during the summer, because the frequency of large immature animals and adults in stage 2 of ovarian development starts to increase after reaching an annual low in late spring (Fig. 2). Concurrently during the summer, the frequency of adults in the advanced ovarian stages (3, 4) declines to an annual low, but then shows an annual rise as oocytes in stage 2 ovaries mature. The new period of oocyte formation continues during the fall. The progression of oocyte formation and development was observed in five individuals marked and subsequently recaptured one to three months later. One animal, whose ovary failed to show an increase in development of oocytes from November 17 to February 24, was injured and ready to molt on the latter date; perhaps the injuries and molting had inhibited oocyte development. The frequency of females in stage 2 declines to the annual low, as maturation of oocytes continues during winter and spring months. The complete seasonal cycle of oocyte development during one year in Shiloh Cave is known for one marked female. This animal had molted a few days before being marked on August 21, 1961, when its ovary

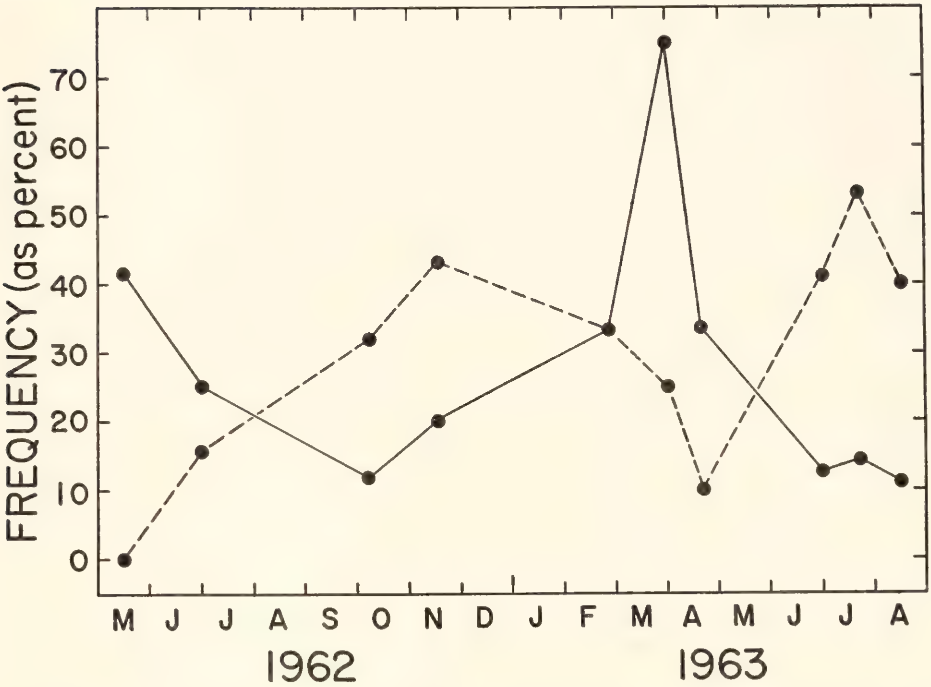

FIGURE 2. The frequency of adult females with the ovary in advanced stages of oocyte development, stages 3 and 4 (solid line), and the frequency of large immature and adult females with the ovary undergoing seasonal proliferation of oocytes, stage 2 (dashed line), in the population of *O. p. inermis* in Shiloh Cave. The number of adult females in samples varied from 4 to 24. The number of adult and large immature females in samples varied from 4 to 32.

was in stage 1. It was in stage 2 on September 23 and was carrying eggs on June 30. The animal was taken to the laboratory (15–17° C.) where it died on August 5. The embryos were nearly ready to hatch and the ovary was in stage 1, completing a one-year reproductive cycle.

Annual changes in the frequency of immature crayfish <18 mm. carapace length present in the population occur as a result of the annual female reproductive cycle (Fig. 1). The frequency of immature animals rises during the summer and early fall as new young leave the adult female. The frequency declines sharply during late fall when new young are no longer added to the population and as larger immature crayfish are becoming adults.

DISCUSSION OF FEMALE REPRODUCTIVE CYCLE

Ortmann (1960) claimed that species of crayfishes living in cool streams where the yearly temperature tends to be uniform lose seasonal periods of reproduction. This is not the case for *O. p. inermis* in Shiloh Cave. My data suggest that eggs are laid and carried during spring and summer months, eggs are hatched during summer and fall months, and that most young enter the stream population during late summer and fall. Reports on the occurrence of ovigerous females during other than spring and summer months do not negate this hypothesis. The developmental time of the eggs in *O. p. inermis* is from two to three months. A female laying eggs towards the end of the egg-laying period could still be carrying them during late fall or early winter.

The time of events from the beginning of oogenesis to hatching of the young during the year is similar in *O. p. inermis* as in many epigean species of crayfishes. But there is a difference in the proportion of females laying eggs during any one year. Nearly all the adult females of *O. propinquus* (Van Deventer, 1937) and *O. immunis* (Tack, 1941) lay eggs each season. I suggest that not all adult females of *O. p. inermis* lay eggs each season, because few ovigerous females are found. The probability of finding ovigerous females is high since there is a long developmental time (2–3 months). Also, a small proportion of young relative to the adult population is found. A small percentage of females laying eggs may be an adaptation to the relatively low food supply in Shiloh Cave and may be a means of regulating population size of these crayfish.

Male Reproductive Cycle

Two morphological types of adult males, known as forms I and II, occur in species of the subfamily Cambarinae. These forms are associated with the reproductive cycle and usually alternate during the life of an individual (Hobbs, 1942). Form I males are in the breeding stage while form II adults are not known to mate. An immature male is in form II until the last juvenile molt when it changes to form I and thereafter alternates between form II and form I, when molting, for the remainder of its life. Form I males of *O. p. inermis* in Shiloh Cave have large, curved hooks on the ischiopodite (third most proximal segment) of the second pair of walking legs and small hooks on the ischiopodite of the third pair of walking legs. Form II males in Shiloh Cave have small, blunt hooks on the second pair and no hooks, except on rare occasions, on the third pair of walking legs. There are also differences between the gonopods (copulatory, first abdominal appendages) of the two male forms. In relative terms both tips of each gonopod are longer and pointed in form I males but shorter and blunt in form II males. In addition one of the tips of each gonopod is corneous (hard and dark in color) in form I males; otherwise the entire distal one-fourth of the gonopods in these males remains white, even after the rest of the animal shows post-molt darkening.

Observations on Male Reproductive Cycle

Data of the occurrence of adult males ($\geq$21.5 mm. carapace length) were obtained during four consecutive years in Shiloh Cave (Fig. 3). The number of adult males sampled varied from 7 to 33. The frequency of form I males is lowest during the summer months, increases and reaches a maximum during the fall, and decreases during the late winter and spring months to the summer minimum. The annual wave form is uniform with respect to time of year but does differ in amplitude of the maximum and minimum. The maximum frequencies recorded during the fall of each year from 1960 to 1963 were 85%, 84%, and 97%, while the minimum frequencies for these years were 50%, 50%, and 47%, respectively. Minimum frequencies of 33% and 27% were recorded for the years 1963 and 1964. Data for the years 1960–63 show a rapid increase in form I males with progression of the fall molting period (see results on molting), but the most abrupt change in frequency was recorded during 1964 when the percentage of form I males changed from a low of 27% on August 16 to 67% by August 26. Most of the adult males are in form I by the beginning of November when mating is probably at its peak. After November the trend reverses and males may begin reverting to form II. During 1960–61 there was a decrease in form I males from 80% on December 10 to 50% on February 14 and during 1962–63 from 97% on November 17 to 57% on February 24. The frequency of form I males was 92% on January 4, 1964. This is the latest record in the annual cycle I have when adult males were predominantly in breeding form before the annual change in the population to form II. It appears that the initial prominent decrease in form I males occurs at the end of January and beginning of February. On October 7 and November 17, 1962, a total of 18 form I males were marked and only three of these were recovered on February 24,

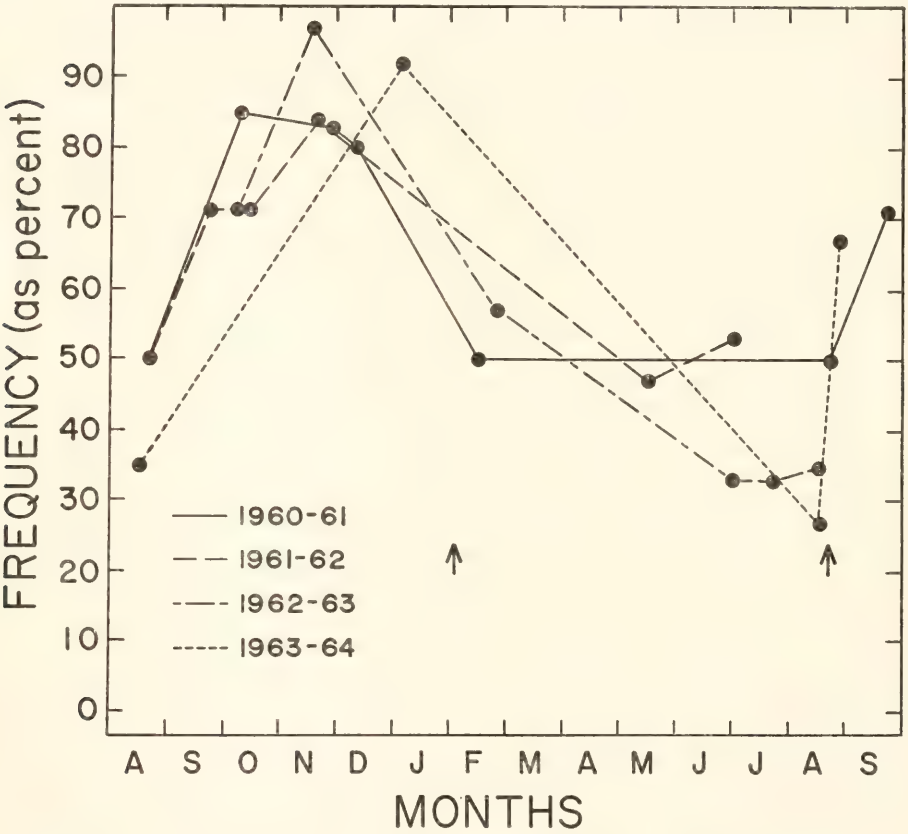

FIGURE 3. The annual change in frequency of form I male, *O. p. inermis* in Shiloh Cave during four successive years. The arrows indicate when the annual frequency decreases in February and increases in August. The number of adult males varied from 7 to 33.

1964. Probably many of the remaining 15 had already changed to form II. After February the frequency of form I males may continue to decrease to the annual minimum. During 1963 the frequency dropped from 57% on February 24 to 33% by June 30 and continued at this level until the annual fall increase of form I males. It is obvious that, while most adult males are in form I during the fall, not all of them revert to form II during the spring.

The testis of a crayfish is a compound acinar organ, trilobed, and lies immediately ventral to the pericardium. In *C. montanus* acini are proliferated annually, produce a quantity of spermatozoa, and then degenerate (Word and Hobbs, 1958). A few testes were preserved during different months from July, 1962, through August, 1963, to determine whether an annual cycle of spermatogenesis occurs in the cave environment. As the number of testes studied was small (20) the results should be considered preliminary and not entirely conclusive.

TABLE I

Summary of annual changes occurring in the testes of adult O. p. inermis in Shiloh Cave and correlation of these changes with morphological form

Months	Predominant male form	Condition of testes
June, July, August	II (change from II to I begins during August)	Testes in full seasonal production of spermatozoa; much meiotic activity; many spermatids; great amounts of transforming spermatids and translocation of spermatozoa to collecting ducts.
October and November	I	Spermatogenesis ebbing; large amounts of spermatids, transforming spermatids, translocation of spermatozoa and degeneration of acini.
February	Annual change from I to II begins	Seasonal spermatogenesis essentially over; testes relatively small due to previous massive degeneration of acini; some spermatids; some translocation of spermatozoa.
March, April, May	II	End of seasonal spermatogenesis; few spermatids or spermatozoa remain.

Apparently a new annual season of proliferation of acini and spermatogenesis begins during the summer months (Table I). Perhaps June was the month of 1963 when meiotic activity was principally initiated. A peak of spermatogenesis occurs during July and August. Seasonal spermatogenesis decreases during the fall and is nearly completed by the end of February when many adult males are reverting to form II. There appears to be little spermatogenic activity during the spring months.

DISCUSSION OF MALE REPRODUCTIVE CYCLE

The population of *O. p. inermis* in Shiloh Cave is similar to many epigean species of crayfishes in having two periods of change in male form during the year. In some species all or most of the adult males in a population change form before and after the mating season: *O. limosus* (Ortmann, 1906), *O. immunis* (Tack, 1941), *O. obscurus* (Ortmann, 1906; Crocker, 1957), *O. propinquus* (Van Deventer, 1937), *C. montanus* (Word and Hobbs, 1958), and *C. longulus* (Smart, 1962). This situation is responsible for the predominance of one form or the other during certain months. However, in *O. p. inermis* not all the males change form twice a year, although the change during the fall is nearly complete. This results in the occurrence of form I males throughout the year—over 90% during late fall and winter and about 30–50% during spring and summer. Aiken (1965) reported a similar result for *O. virilis.* Some authors have interpreted data showing both male forms of a species occurring during most of the year to mean that no annual periods of reproduction occur in that species. Such an interpretation does not apply to *O. p. inermis* in Shiloh Cave.

The time of events in the annual spermatogenic cycle during the year appears to be similar, in some respects, to that described by Word and Hobbs (1958) for the epigean species, *C. montanus.*

The Annual Molting Cycle

Previous knowledge of molting cycles in cave crayfish is meager. Hay (1903) inferred from limited observations that an ecdysis of *O. p. pellucidus* occurs in Mammoth Cave, Kentucky, about the same season as in epigean crayfish. Banta (1907) believed that *O. p. inermis* from Indiana molted from two to four or five times a year, depending on size. Putnam (1877) observed a specimen from Mammoth Cave molt twice during six months in the laboratory, although several appendages had been injured previously. Molting in cave crayfish is more difficult to assess than reproductive activities because in addition to effects of light, darkness and temperature on molting in crustaceans, starvation may inhibit molting (Roberts, 1957), multiple leg autotomy sometimes promotes molting (Passano and Jyssum, 1963) and molting is inhibited in ovigerous females (Tack, 1941). For the present analysis of molting in cave crayfish the discussion will be limited to adult and large immature animals.

Observations on Annual Molting Cycle

The best criterion of molting in male crayfishes of the subfamily Cambarinae is the change in ratio of the form of the adults. There are two such changes each year in the Shiloh Cave population (Fig. 3). One occurs principally during February and March (the spring molting period) and the other principally during August and September (the fall molting period). Relative hardness and darkness of the exoskeleton also proved to be valuable criteria in studying the molting cycle of cave crayfish. Pooled data from these sources show the same two annual periods of molting for both sexes (Fig. 4) that were discovered for males from the changes in ratio of male form. These two periods are the predominant times of molting, but some adult and large immature animals are found molting during other months of the year (Fig. 4). The spring molting period is more pronounced in males than females because many of the adult females probably bypass this period and do not molt. The fall molting period is well pronounced in females, and most of them probably molt at this time.

Although both periods are well demonstrated in males, it should be emphasized that these are phenomena of the population as a whole and do not indicate that every male molts twice every year. Small and medium-size animals perhaps do,

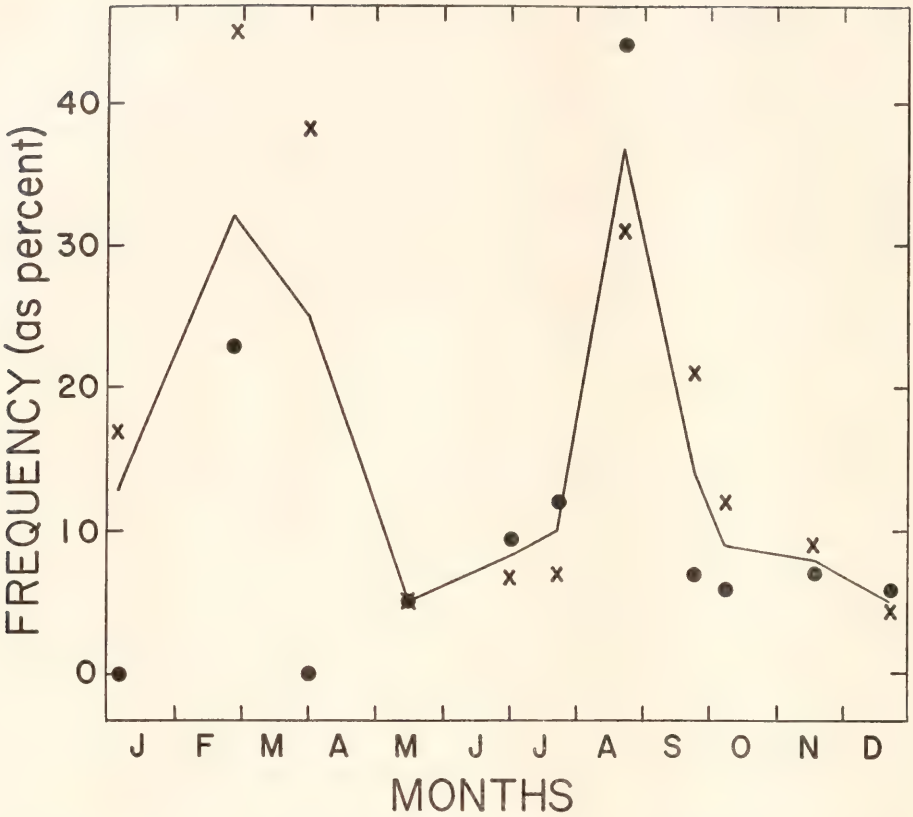

FIGURE 4. Annual molting periods of large immature and adult crayfish, *O. p. inermis*, in the Shiloh Cave population. Data obtained during the years 1962, 1963 and 1964 have been used to construct this graph. The number sampled varied from 12 to 76. The points for each date represent pooled data from the various indicators of molting (ecdysis, relative hardness and relative darkness of the exoskeleton). Females (●), males (×), males and females combined (——).

but it is clear that the larger animals do not. Some large form II males were found during the winter intermolt period, indicating that they had bypassed the fall molting period; but some large form I males with hard, dark exoskeletons occurred during the summer intermolt period, and these animals probably had not molted since the previous fall.

DISCUSSION OF ANNUAL MOLTING CYCLE

In crayfishes living in the central and northern United States the two molting periods are usually less than four months apart, thereby creating short summer and long winter intermolt periods. During one year of Van Deventer's study of *O. propinquus* both molting periods occurred within one month's time. In Shiloh Cave crayfish the two annual peaks of molting of adult and large immature animals are six months apart, with intermolt periods of three months. All of the present data indicate that the peaks of molting occurred at the same times of the year during this study but with differences occurring in the frequency of molting on the same date during different years.

General Discussion

It is clear that the cave-dwelling crayfish *O. p. inermis* has annual cycles of reproduction and molting. The fact that some molting does occur throughout the year suggests that these cycles, at least the molting cycle, are not completely controlled by a seasonal factor in the environment. But something does induce the majority of the animals to molt during two periods of the year and induces the majority of adult males to be in breeding form during the fall. Perhaps a basic endogenous rhythm is present but some seasonal factor in the environment is acting to synchronize molting and reproduction, although imperfectly, in the population. Is there a factor, in the absence of photoperiod and large temperature changes in the environment, which is seasonally variable enough to account for these consistent annual periods?

Gradual changes in organic material or water level, or the occurrence of flooding (rapid changes of water level, increased current and increased turbidity) perhaps could act to synchronize or modify biological cycles in Shiloh Cave, but no quantitative data on the first two variables were obtained. According to Ginet (1960) dissolved organic material is greatest in French caves during late summer and early fall and lowest during the winter. Water levels in seven caves of the central United States are highest during late winter and early spring (Poulson, 1964). However, these may be subtle factors depending on the particular cave system. The occurrence of flooding in caves is well known; in Shiloh it has been observed on December 18, 1961, March 31, 1962, and March 31, 1963—the latter date was near the end of an unusually heavy flood, with high water level continuing through April. Flooding is certainly an adverse environmental factor for rearing of young crayfish, and selection might favor egg-laying and hatching at times other than the possible late November to April flooding season in Southern Indiana caves. The most favorable period for egg-laying in cave crayfish would be towards the end of the flooding season as this would allow time for development and growth of the young to a point where they could cope successfully with the rushing water of the next flooding season. This appears to be the situation in Shiloh Cave and apparently in several other caves where some data are available.

A season of particularly strong flooding perhaps would induce many females either to resorb their oocytes or to lay eggs in a relatively short period of time. This may have been the case during the spring of 1963 when the frequency of females with large, yolky oocytes dropped sharply, reaching 0 by July 21 (Fig. 1). On the other hand, after a season of weak flooding one might expect to find less of an inducing effect on the females and a subsequent lengthening of the period when females either resorb oocytes or lay eggs. During the 1961–62 reproductive cycle weak floods were noted on December 31 and March 31. The decrease in frequency of females with large, yolky oocytes was much slower than in 1963 and was prolonged through the summer and into the fall, finally reaching 0 during September (Fig. 1). The egg-laying period was thereby shortened during 1963 in reference to the period during 1962.

An environmental factor that regulates the annual cycle of gametogenesis and molting in male cave crayfish is unknown. In the Shiloh Cave population each molting cycle is approximately six months in duration; each molt period and each intermolt period have durations of about three months. The spring molting period occurs during the time when flooding may occur, which superficially would appear to give the animals considerable disadvantage in the struggle to survive a severe flood. Observations on March 31, 1963, show clearly that they can molt and survive during a flood. There is no obvious change in the environment to account for the increase in molting and change in male form during August of each year. A possible explanation of such a phenomenon is that the annual cycles of these males are synchronized by the environment some time before August, perhaps by the spring floods.

Annual reproductive periods have been reported for other cavernicolous animals. The cave fish, *Amblyopsis spelaea*, living in the same limestone area as *O. p. inermis*, lays eggs during high water from February through April; the young leave the maternal gill cavity and appear in the streams during late summer and early fall (Poulson, 1963). *A. spelaea*, then, is not dissimilar in its season of production of young from *O. p. inermis*. In France the amphipod *Niphargus orcinus virei* carries eggs throughout the year, but there is a maximum period during the late spring and summer (Ginet, 1960). Ginet concludes that an endogenous rhythm of reproductive activity not directly bound to fluctuations in the environment may be present. This suggests to me that the cycles of reproductive activity of individual amphipods are imperfectly regulated by the environment, with the consequent poor synchronization of reproduction in the population and much overlapping of the individual cycles. A lack of seasonal reproduction has been reported for several cavernicolous animals. Deleurance and Deleurance (1964) found that the proportion of ovigerous female beetles of the subfamilies Bathysciinae and Trechinae shows annual variations, but the variations are not seasonal. Mitchell (1965) found recently pupated beetles, *Rhadine subterranea*, at all times of the year, indicating that seasonal reproduction is absent. Both studies do not show reproductive activities for individuals. Individuals might well have an annual rhythm of reproduction but if the environment does not induce individuals to reproduce seasonally, reproduction will probably occur in a population throughout the year because of individual variation in length of the annual cycles. Perhaps flooding, which may have an effect on seasonal reproduction of aquatic cave animals, has no effect on reproduction of the terrestrial animals.

This study comprised part of a dissertation submitted in partial fulfillment of the requirements for the Ph.D. at the University of Illinois. I am deeply indebted to Dr. Michael J. Greenberg for his assistance and guidance during this study, to Dr. R. A. Brandon, Mr. Jack Donahue and Mr. Warren Brigham for assistance on field trips to the cave, and to Mr. W. R. Day for permission to use Shiloh Cave. I am thankful to Dr. T. L. Poulson for his assistance in preparation of this manuscript.

SUMMARY

1. Annual periods of reproduction and molting of *Orconectes pellucidus inermis* in Shiloh Cave, Indiana, have been observed. Both sexes of *O. p. inermis* in Shiloh

Cave appear to initiate a new annual period of gametogenesis in early summer. The number of adult females whose ovary contains large, yolky oocytes is declining to an annual minimum and the majority of adult males are in form II. Eggs are being laid and hatched and young crayfish are leaving the adult and entering the stream population. Oocyte formation and growth, spermatogenesis, and maturation of spermatozoa continue during the fall months. Many adult and some large juvenile males molt to form I. During fall months most adult males are form I, they have large supplies of spermatozoa, and mating presumably occurs. Oocytes of some females begin to mature and there is an increase in the proportion of females having the ovary filled with large, yolky oocytes.

During late winter and early spring months spermatogenesis and oogenesis decline to an annual minimum, that is reached during the spring months. Concurrently many adult males molt to form II. Not all the adult females molt at this time of year. The proportion of females with large, yolky oocytes in their ovaries increases to the maximum during the spring.

2. Evidence for environmental synchronization of the reproductive and molting cycles of individuals in the population is discussed.

3. Occurrence and absence of seasonal periods of reproduction reported by other investigators in a few species of cavernicolous animals are discussed.

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STUDIES ON THE ELASMOBRANCH KIDNEY. IV. THE SECRETION OF PHENOL RED BY THE SMOOTH DOGFISH, MUSTELUS CANIS[1]

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While the reabsorptive process of the kidney of the smooth dogfish has been studied in relation to urea (Kempton, 1953), tubular secretion has not been examined. The present study was initiated to determine to what extent these renal tubules are capable of secretory activity.

A further consideration is the fact that in recent years increasing use of *Mustelus canis* as an experimental animal has resulted in one body of information concerning the smooth dogfish and another for the spiny dogfish (*Squalus acanthias*), with little work being done on both species. Although the similarity in popular names suggests a close relationship between the two animals, they are in fact placed in different primary subdivisions of the sharks (Bigelow and Schroeder, 1948). Since phenol red elimination has been studied in the spiny dogfish by Willie Smith (1939) a study of this process in the smooth dogfish permits comparison of the two species.

MATERIALS AND METHODS

Female smooth dogfish weighing 3.5 kg. or more were kept untreated in running sea water for approximately 24 hours after their capture by otter trawl. This gave time for recovery from the effects of capture and transportation. On the day preceding their use they were weighed and injected intravenously with inulin (10 ml./kg. of a 10% solution freshly prepared with distilled water). The following morning an in-lying catheter was tied into the urinary papilla, a balloon was attached to collect urine, a blood sample was taken and the animal was injected with phenol red. The usual dosage was 5.0 ml./kg. of an 800 mgm.% solution in 1% $NaHCO_3$ administered intravenously through the caudal vessel. A period of two to three hours elapsed before starting the collection of urine, in order to insure sufficient time for distribution of the dye. In a few cases the dosage was greater, the largest being an intravenous injection of 5 ml./kg. of a 2400 mgm.% solution of phenol red, accompanied by 2.0 ml./kg. injected intramuscularly. Even the standard dosage gave a deep red color which disappeared gradually over 12–36 hours. Repeated collection periods were made until the blood level of phenol red was too low for accurate determination or some untoward event caused the termination of the experiment. Usually the periods were 3–4 hours in duration, except for one 6–8-hour period overnight. For each period the plasma level of phenol red and inulin was taken as the average of the concentrations at the start and end of the period. Urine was collected with the aid of abdominal massage.

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[1] Analysis of the data was carried on at the Research Laboratory of the Marine Studios, Marineland, Florida, which was made possible by the hospitality of Marine Studios and by the interest of Curator Clifford Townsend. The time was provided by a Faculty Research Fellowship awarded by Vassar College, and the expense was aided by Grant No. 3521—Penrose Fund (1964) of the Philosophical Society of Philadelphia.

The method of collecting urine and the analytical method for inulin were as described previously (Kempton, 1953). Phenol red determinations were made in a Coleman Junior Spectrophotometer at 560 millimicra, after the blood and urine were diluted to range with 5% $NaHCO_3$. At lower phenol red concentrations even a very small amount of scatter would cause significant error. Such scatter was compensated by use of a blank consisting of plasma which had been collected previous to dye injection, and which had been diluted with $NaHCO_3$ solution to the same extent and at the same time as the plasma containing phenol red. With higher plasma concentrations, and with urine, the blank was merely 5% $NaHCO_3$. Suitable phenol red standards were prepared in 5% $NaHCO_3$.

During the summers of 1962 and 1963 all the animals were kept at the temperature of the sea water system at the laboratory, namely 20° C. ± 2°; in the latter part of the summer of 1964 some of the animals were kept in running sea water which had been chilled to between 12° C. and 16° C. In this colder water the fish had a markedly lowered rate of urine flow.

A total of 100 successful collection periods was obtained over the three summers. Clearances were calculated from the volume of urine flow and the concentration of phenol red and inulin in the plasma and urine. Other calculations were made to determine possible causal relationships.

In making some of these computations it was desirable to take into consideration the binding of phenol red to plasma proteins. Pooled samples of heparinized smooth dogfish blood were separated by centrifugation. A phenol red stock solution was prepared by dissolving dry phenol red in some of this plasma. This was added to other aliquots of plasma in varying amounts to give concentrations of phenol red in approximately the same range as that found in the experimental animals. Some of the dye-free plasma was retained for use in blanks during the determination of phenol red concentrations. Each dye-containing plasma sample was filtered under oil through a dialyzing membrane[2] at a negative pressure of 120–130 mm. Hg, the filtrate being collected under oil. The dye concentration of the plasma was compared with that of its filtrate. The average of 15 determinations indicated that 89.3% of the plasma phenol red is filterable in the range of experimental plasma levels. This value was used in the calculations of the rate of phenol red filtration. Since this factor as applied produces only a 9% reduction in the calculated filtration rate, any small error in the value would have little significance in the computations.

RESULTS AND DISCUSSION

When the total phenol red excretion per ml. of filtrate was plotted against plasma concentration (Fig. 1), the excretion rate increased rapidly as the plasma levels increased. In the graph tubular secretion is represented by the distance between a circle on the upper curve and the corresponding square on the lower; the amount

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[2] Arthur H. Thomas Co., Philadelphia, catalogue no. 1165–A2.

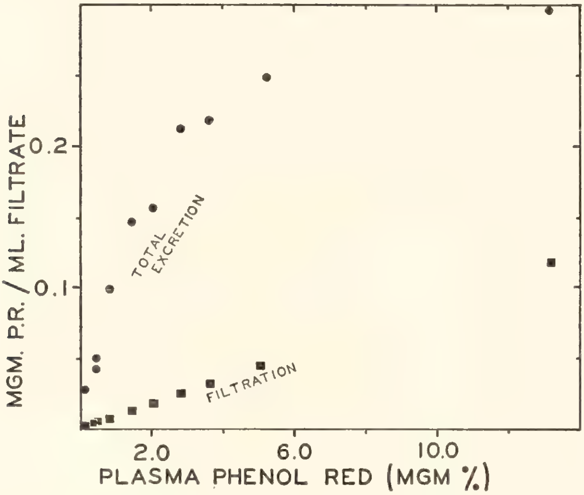

FIGURE 1. Relation between varying concentration of phenol red in the plasma and the amount of dye excreted per milliliter of filtrate. In this and other figures each point represents an average of 10 collection periods, based on increasing plasma phenol red concentrations. Circles = total excretion; squares = calculated filtration.

of dye filtered is represented by the distance from the square to the baseline. Tubular secretion thus became roughly constant at plasma levels above 2–3 mgm.%. The ratio between the clearances of inulin and phenol red is shown in Figure 2. Since the clearance of inulin represents the rate of filtration, ratios above unity depend on tubular secretion. Thus, there was a significant amount of secretion at all plasma levels, but it was especially great at the lower concentrations. The fraction of the excreted dye which was secreted by the tubules (Fig. 3) ranged from an average of 94.8% in the 10 periods in which the plasma phenol red was

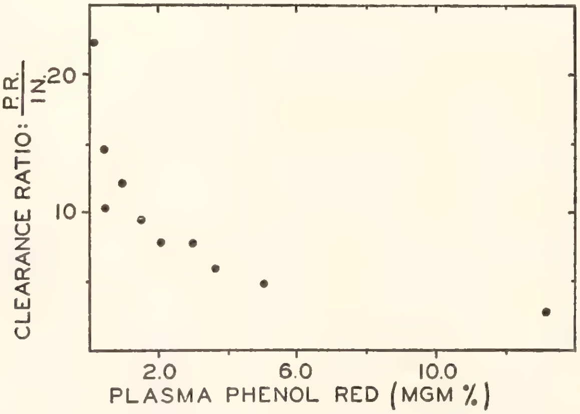

FIGURE 2. Relation between varying concentration of phenol red in plasma and the ratio of the clearances of inulin and phenol red.

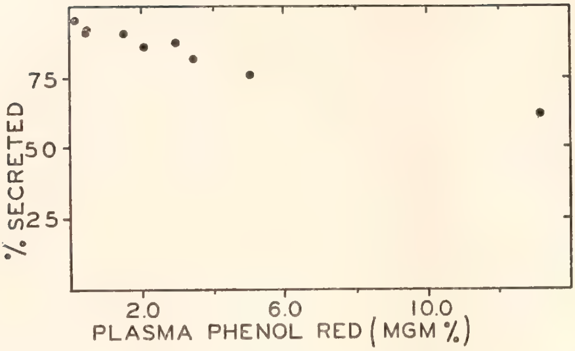

FIGURE 3. Percentage of the excreted phenol red which was eliminated by tubular secretion at varying plasma levels.

lowest, to an average of 61.7% in the 10 periods in which the plasma load was greatest. As the plasma level of phenol red was increased, the fall in the secreted fraction was essentially linear.

Relating excretion to units of filtrate as above eliminates the effects of variation in the volume of filtrate. The absolute rate of secretion (mgm./hr./kg.) was much more variable. At low plasma concentrations the amount of secretion rose rapidly with increased levels of phenol red (Fig. 4), reaching a maximum secretory rate when the plasma phenol red was between 2 and 3 mgm.%. However, this maximum rate was not maintained as the plasma level increased, but fell to a little less than 50% of the maximum. This will be discussed below.

The amount of secretion of phenol red was sufficiently great to lead to a very high urine/plasma (U/P) ratio when the plasma levels were low, and this ratio fell rapidly as the plasma levels increased (Fig. 5). Since the decrease in tubular

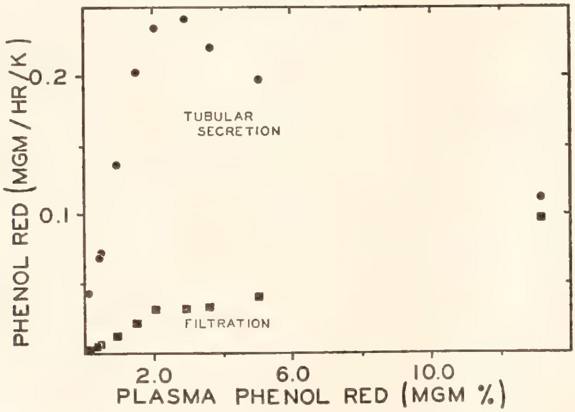

FIGURE 4. Total amount of tubular secretion of dye and of filtration at varying plasma concentrations. Above a plasma level of 2.0 mgm% the filtration rate was less than would have been expected on basis of unchanged filtration volume. Circles = tubular secretion; squares = filtration rate.

secretion appeared only at plasma levels above 2–3 mgm.%, it is clear that the first part of the fall in U/P ratio was not dependent on decreased secretion.

The U/P ratio of inulin indicates the degree of concentration due to the reabsorption of water from the filtrate. Although there was a very gradual fall of nearly 50% in the inulin U/P ratio, starting at a phenol red plasma level of 2–3 mgm.%, the U/P ratio of phenol red was obviously of a different order of magnitude; and unlike the inulin U/P value it was very responsive to changes in plasma phenol red level.

The interpretation of the data is obscured by the fact that in these experiments the rate of filtrate formation (inulin clearance) was depressed at the higher phenol red levels. The cause of this is not clear, but it could be due to the high phenol red levels, to the large amount of $NaHCO_3$ in the vehicle for phenol red, or to the lower water temperatures which prevailed during several of the periods. Regardless of cause, this depression is seen in Figure 6, in which the filtration rate (inulin clearance) values are somewhat scattered but clearly became lower when the plasma levels of phenol red were above approximately 2 mgm.% The depression of total water reabsorption, represented in the same figure, is considered to be largely a consequence of the reduced filtration. It is clear from the data illustrated in Figure 7 that a relatively constant proportion of the filtered water was reabsorbed. This fraction varied only from a minimum of 60% to a maximum of 75%, the variations following no marked trend. In contrast the amount (ml./hr./kg.) of water reabsorbed and the amount of urine produced (Fig. 6) were much more variable than this.

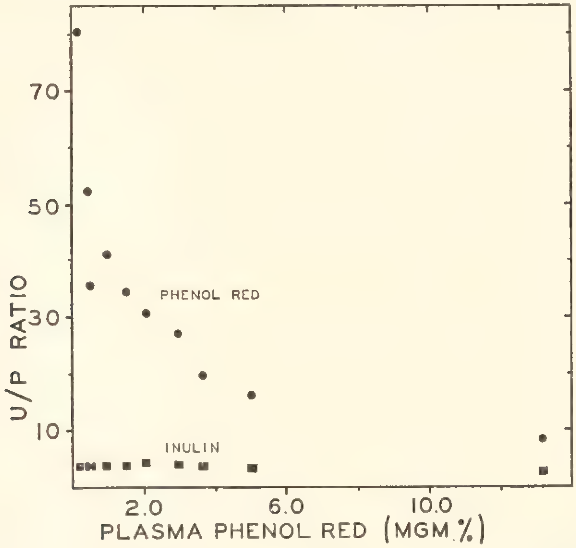

FIGURE 5. U/P ratios of phenol red and inulin at varying plasma phenol red levels. Circles = phenol red U/P; squares = inulin U/P.

The observation that at plasma levels higher than approximately 3 mgm.%

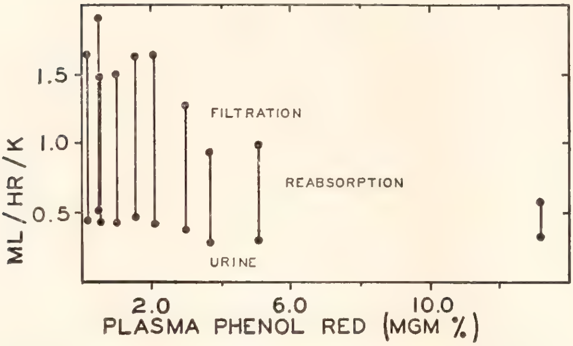

FIGURE 6. Volume of filtration, reabsorption and urine flow at varying plasma levels of phenol red.

phenol red there was a fall (Fig. 4) in total secreted phenol red (mgm./hr./kg.) raises a question as to its nature. It is clear that there was also a fall in the rate of formation of filtrate. Not only did this appear in the inulin clearance ("filtration") of Figure 6 but logically it would have to be true even though that evidence were not available. This follows from the fact that with increasing plasma levels the phenol red concentration in the filtrate automatically becomes greater and it is only through reduction in volume that the total amount of filtered dye could be decreased as plasma levels rise.

A reduction in the rate of filtrate can result from two different causes, acting separately or together. First, decreased total filtration could result from a reduction in the filtration by the individual glomeruli (perhaps as a result of reduced intracapillary pressure). Alternately, the same result could be obtained by a complete cessation of function of some glomeruli while others continued with unimpaired activity. The data appear to give a basis for choosing between these two explanations of the present results, because the two possibilities have different sequelae.

If the reduced total filtration were due to a reduction in filtration of all individual glomeruli, there would be several consequences. (1) There would be a reduction in the total amount of filtered dye. Figure 4 shows that this was the

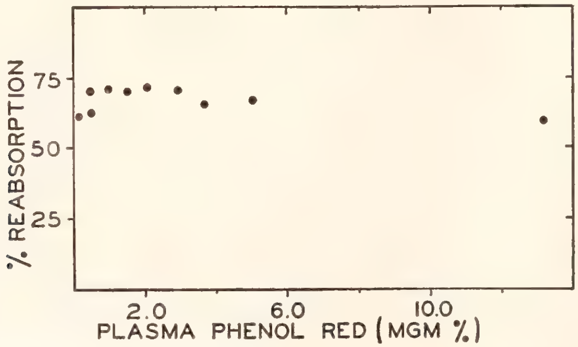

FIGURE 7. Percentage of water reabsorbed from the filtrate at varying plasma phenol red levels.

case, in that the increase in dye filtration was inadequate to keep pace with the expectations based on the increasing concentration of dye in the plasma and filtrate. (2) In the reduction in the total volume of urine-plus-reabsorption, changes of reabsorption obviously cause changes in urine rate. Thus, in Figure 6 while the filtration rate continued to fall with increased phenol red plasma levels, a reduction in the water reabsorption kept the urine rate from falling to even lower levels. (3) The effect that decreased glomerular filtration would have on total phenol red tubular secretion depends on whether the cells were secreting maximally. Since the fall in filtration which is being considered took place at or above levels at which tubular secretory maximum had been reached (Fig. 8), discussion can be confined to this special case. (4) With the cells secreting maximally, a slower rate of progress along the tubule should lead to higher rather than lower U/P ratios, since more time would be available for the higher concentration to be reached. In the event that the concentration ratio should become the limiting factor in secretion, there would be an upper limit of concentration ratio, but the ratio should not fall.

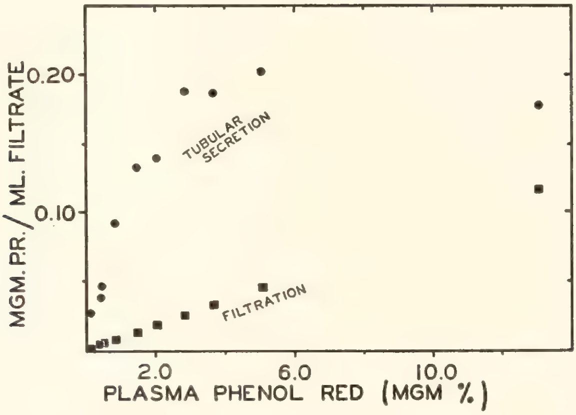

FIGURE 8. Tubular secretion related to unit volume of filtrate at varying plasma phenol red concentrations. Circles = tubular secretion; squares = filtration.

In fact the ratio did continue to fall in a consistent fashion (Fig. 5) which gives no indication of an upward tendency.

With the other possibility (complete cessation of function of some glomeruli, and continued unchanged function of the others) the effect on total filtration volume and total phenol red filtration would be no different from the first case. But there is a clear difference between the two situations. If the functioning glomeruli were unimpaired, decreased filtration and consequent decrease in the amount of water reabsorbed should have no effect on the percentage which is reabsorbed. This is because each glomerulus and tubule which is still functioning is behaving as it did previously, producing and processing the original amount of filtrate. As a matter of fact, the percentage of water reabsorption remained surprisingly constant (Fig. 7). When phenol red is considered, it is clear that the filtrate which formed under these conditions would contain an unchanged concentration, but a reduced total amount of phenol red would be filtered in view of the decreased number of filtering glomeruli. In those tubules whose glomeruli were still functioning, the processes of filtration, reabsorption and secretion would be unchanged. The non-functioning glomeruli and their tubules would simply be subtracted from the picture. Therefore there should be no change of U/P ratio related to the change in rate of filtration. The smoothness of the curve in Figure 5 shows this to be the case.

Another consideration also points toward the elimination of functional glomeruli as the cause of both the reduced total secretion of phenol red, and the reduced glomerular filtration. If secretion is considered in terms of mgm./hr./kg. there is a marked fall in secretory rate with plasma levels above 3 mgm.% (Fig. 4). On the other hand, if secretion is expressed in terms of each milliliter of filtrate produced, this fall either does not appear or is very much reduced (Fig. 8). The explanation appears to be that the absolute amount of secretion is the total of all the glomeruli and tubules, whether active or inactive. But when secretion is considered in relation to filtrate volume, only the active glomeruli and tubules are being considered, and these still active tubules continue to behave in an unchanged manner. Thus, the difference between Figures 4 and 8 offers evidence that the reduction in filtration is due to a reduced number of glomeruli rather than to reduced function of individual glomeruli.

One further consideration points in the same direction without involving phenol red. Figure 6 indicates that above the critical phenol red plasma concentration of 2–3 mgm.% the rate of filtration falls about 50%, urine production falls about 50%, total reabsorption of water falls about 50%; while on the other hand the *percentage* of water which is reabsorbed falls very little (Fig. 7). Such an agreement is compatible with a situation in which some of the glomeruli and tubules are removed from the over-all function while the remainder continue to behave as before. No other explanation seems to fit the facts with equal cogency.

A comparison of the data derived from these experiments with some of those found by Smith in the spiny dogfish, is summarized in Table I:

TABLE I

Comparison of the smooth dogfish (Mustelus canis) and the spiny (Squalus acanthias) (Smith 1939)

	Smooth	Spiny
Phenol red clearance (cc./kg./day)	593	1750
% phenol red secreted	91.7%	99%
$\frac{\text{Clearance phenol red}}{\text{Clearance inulin}}$	14.8	22.5
Tubular maximum mgm./kg./day	5.8	18.1
Inulin clearance (cc./kg./day)	40	78

It seems clear from these data that the general behavior of the kidneys of the two species is similar. At plasma dye levels below 1 mgm.% the phenol red clearance, the percentage of the excreted dye which is actively secreted by the tubules, and the ratio of the clearances of inulin and phenol red are all somewhat lower in the smooth dogfish than in the spiny. The tubular maximum, which seems to be reached at about the same plasma level in the two species (2–3 mgm.%), is lower in the smooth dogfish. The fact that in all these features the values in the smooth dogfish are lower than in the spiny suggests the possibility that the relation between the mass of effective kidney tissue and total weight may be somewhat different in the two animals, the larger (smooth dogfish) on this basis having less excretory tissue in relation to the whole.

SUMMARY

1. With increasing plasma concentrations of phenol red, starting at very low levels, there is a rapid and extensive increase in its excretion, reaching an asymptote at approximately 10–11 mgm.%. This is clearest when the data are so treated as to neutralize the effect of variations in the rate of filtration.

2. Much more phenol red is secreted by the tubules than is filtered by the glomeruli. The percentage of excreted dye which was eliminated by tubular secretion varied from a maximum of 96% at the lowest measurable plasma levels to a minimum of about 60% at the highest blood levels obtained. The decrease was essentially linear.

3. At low plasma levels the ratio of the clearance of phenol red and inulin was approximately 25; at the highest plasma levels the phenol red clearance was reduced to only about three times that of inulin.

4. The U/P ratios were of a very different order of magnitude. That of inulin remained relatively constant at a level between 2 and 5; while with increasing plasma levels the phenol red U/P ratio fell from an initial 60 to a minimum of approximately 6. A fall below 1.0 has been reported for the spiny dogfish but this was at plasma phenol red levels far above those achieved in the present experiments.

5. In this series of experiments the rate of phenol red filtration and the rate of phenol red secretion both became reduced at the higher blood levels of phenol red.

6. With decreasing rates of filtrate formation, a reduction in the rate of water reabsorption prevented rates of urine formation from falling to even lower levels than they did.

7. While at higher plasma levels of phenol red there was a decrease in total water reabsorption, there was very little change in the percentage of the filtrate which was reabsorbed.

8. Data produced by these experiments indicate that the reduced filtrate formation at higher plasma levels was due primarily to a complete cessation of the function of some glomeruli rather than to a mere reduction of filtration rates of all the glomeruli.

9. Comparison of these data on *Mustelus* with those existing in the literature on *Squalus acanthias* indicates that the two kidneys behave in much the same fashion. The fact that most measurable factors were relatively lower in *Mustelus* suggests that the total excretory mass may be smaller in relation to the total weight in this animal. It is possible also that some of this difference might disappear if the two species were compared on the basis of body surface rather than body weight.

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EXCYSTMENT OF CRYPTOCOTYLE LINGUA METACERCARIAE[1]

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Previous investigations show that metacercariae of several species of trematodes will excyst after treatment with digestive enzymes (Faust and Khaw, 1925, 1927; Stunkard, 1930; Ferguson, 1940; Hunter and Chait, 1952; Hoffman, 1959), or after a change in physical conditions (Bacha, 1960; Dawes, 1961). Specifically the role of bile has been investigated (Oshima, Yoshida and Kihata, 1958; Oshima and Kihata, 1958; Kobayashi *et al.,* 1959; Wickerhauser, 1960), but other factors involved in metacercarial excystment are mostly uncharacterized. The process of liberation from enclosing membranes among larval cestodes (Rothman, 1959) and larval nematodes (Rogers, 1962) is more completely documented. Factors influencing the activation of an acanthocephalan cystacanth have been reported by Graff and Kitzman (1965).

Experiments presented here were undertaken to determine specific factors involved in the escape from the primary cyst by the metacercaria of *Cryptocotyle lingua* Creplin 1825, a parasite of fish-eating birds and mammals. *In vitro* studies were carried out under conditions simulating those known to exist within the lumen of the alimentary tract of potential vertebrate hosts.

MATERIALS AND METHODS

Metacercariae were collected from the skin and fins of the cunner, *Tautogolabrus adspersus* (Walbaum), taken from waters near Woods Hole, Massachusetts, by the Marine Biological Laboratory Supply Department and myself.

Tissues containing cysts were cut into small pieces, covered with MBL Formula sea water (Cavanaugh, 1956), and macerated for one minute in a Waring Blendor. Primary cysts were recovered intact and completely free of host tissues.

The cysts were collected in a capillary pipette with the aid of a dissecting microscope, washed twice in saline, and distributed into 5-ml. covered stender dishes (10–20 cysts per dish). The liquid was drawn off and 2 ml. of the medium to be tested were added. Cysts used in the carbon dioxide atmosphere studies (5% CO_2–95% N_2) were placed in Warburg flasks which were gassed 5 minutes before being closed.

The medium consisted of Locke-Ringer (without dextrose) in which the substance to be tested was dissolved. A pH of 7 or pH 2 (pepsin solutions) was maintained in all experiments, except where otherwise stated. Experiments were performed at room temperature (22–26° C.) or at 37° C. At 37° C., the test

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[2] NSF Postdoctoral Fellow.

TABLE I

Relationship of enzyme concentration, pH, and temperature to excystment in pepsin

Pepsin concentration	pH	Temperature (°C.)	No. of cysts	No. excysted in 90 min.	%
Saturated	1	22–26	50	8	16
		37	50	23	46
1%	1	22–26	13	1	8
		37	13	3	23
1%	2	22–26	83	4	5
		37	83	8	10
1%	3	22–26	63	0	0
		37	458	11	2
1%	4	22–26	13	0	0
		37	13	0	0

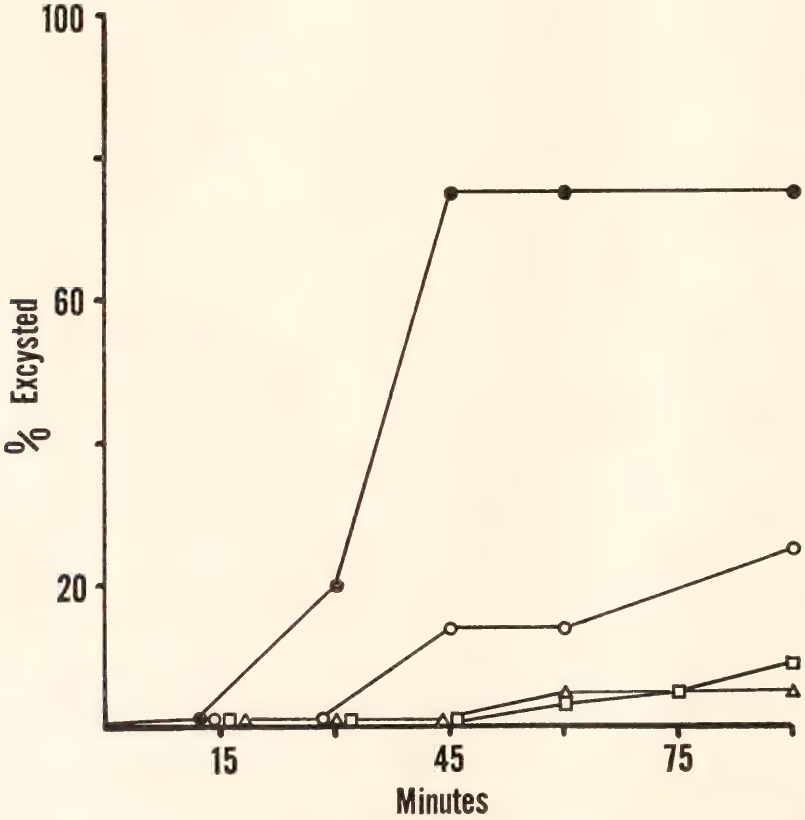

FIGURE 1. Excystment without gastric pretreatment. Conditions: pH 7 at 37° C. △—△ 1% trypsin plus gull bile (dil. 1:9), □—□ 1% trypsin plus 0.1% sodium taurocholate (NaT), ○—○ 1% trypsin plus 0.1% NaT and 0.01 *M* cysteine, ●—● gull intestinal juice (dil. 1:4).

TABLE II

Effect of 1% pepsin pretreatment (pH 2) on excystment in 1% trypsin (pH 7)

Minutes in pepsin	No. of cysts	No. excysted in 30 min.	%
5	30	0	0
10	60	5	8
20	30	5	17
30	320	150	47
40	50	23	47

solution and the dish were warmed before the larvae were introduced. Observations were made with a dissecting microscope. The worms were considered excysted when the enclosing membrane had been breached, but not necessarily sloughed.

Pancreatin and pepsin (powder, N.F.) were obtained from Fisher Scientific Company; trypsin (1:110) from Pfanstiehl Laboratories. Sodium taurocholate was a product of Mallinckrodt. L(+)cysteine hydrochloride was obtained from Matheson, Coleman and Bell.

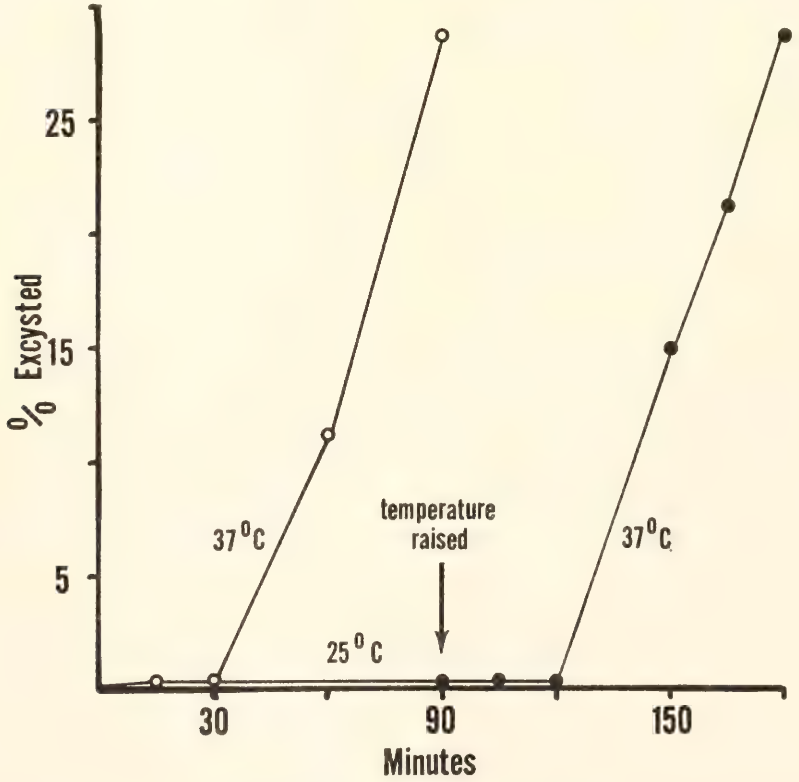

FIGURE 2. The effect of temperature on excystment. Conditions: 1% trypsin plus 0.1% sodium taurocholate and 0.01 M cysteine (pH 7).

RESULTS

Temperature and pH changes, alone or sequentially applied, had no direct effect on excystment. Excystment did not occur in Ringer at pH 1 (Ringer-HCl) or pH 7 at room temperature or 37° C. during a 10-hour period, nor during 5 hours at pH 7 after as much as 5 hours pre-exposure to pH 1. Saturated pancreatin, 1% pancreatin, 1% trypsin, 0.1% sodium taurocholate and 0.1 *M* cysteine had no effect when tested singly. The response with pepsin depended upon its concentration, pH and temperature (Table I).

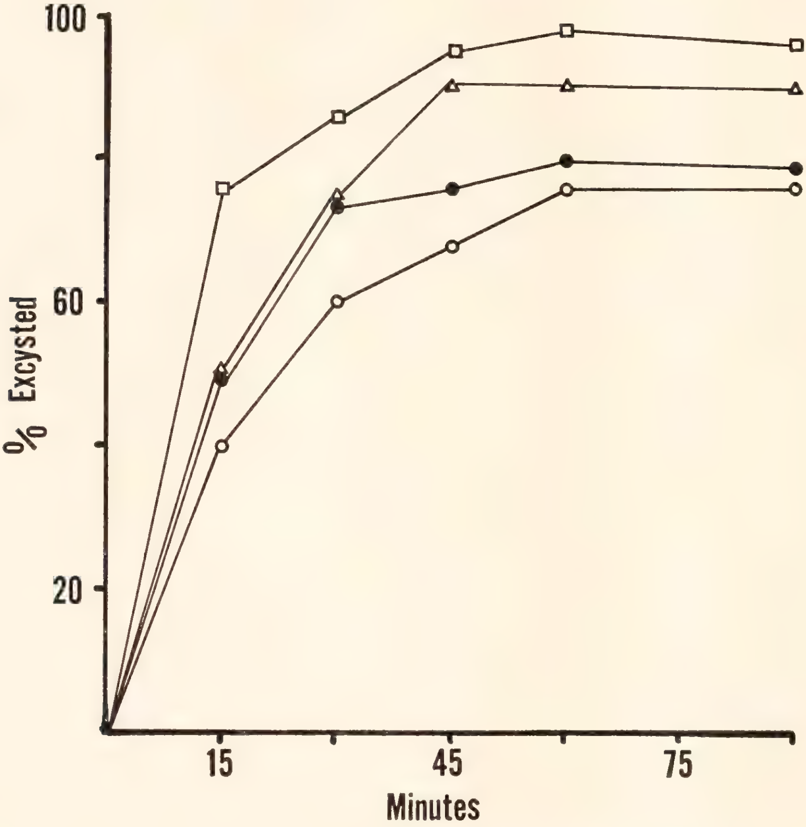

FIGURE 3. Excystment after gastric treatment. Conditions: 1% pepsin (pH 2) for 30 minutes at 37° C., removed to 1% trypsin plus 0.1% sodium taurocholate at pH 7 (○—○), ●—● plus 5% CO_2 atmosphere, □—□ plus 0.01 *M* cysteine, △—△ plus 0.01 *M* cysteine and 5% CO_2 atmosphere.

Some excystment was initiated by exposure to conditions of the small intestine without pretreatment with gastric conditions (Fig. 1). Natural fluids from *Larus argentatus* (herring gull) were tested also. Several dilutions (1:4, 1:9, 1:14) of fresh bile were not effective, but incubation in intestinal juice led to 75% excystment within 45 minutes (Fig. 1). Incubation at room temperature failed to induce excystment within a physiologically significant period, but at 37° C. the usual course of excystment was observed (Fig. 2).

Pretreatment with gastric conditions followed by those of the small intestine proved most effective in stimulating excystment. Again, this effectiveness was not related directly to temperature or pH. Pretreatment at pH 2 (Ringer-HCl) for 30 minutes failed to stimulate excystment in 1% trypsin, and excystment did not occur in Ringer at pH 7 after 30 minutes pretreatment in 1% pepsin. Pretreatment with pepsin initiated excystment in trypsin (Table II), and enhanced the rate of excystment in trypsin with other substances and under different physical conditions (Fig. 3). Maximum excystment (98.1%) was attained within 60 minutes in trypsin-bile salt with cysteine as reducing agent. A carbon dioxide atmosphere was not required for maximum excystment.

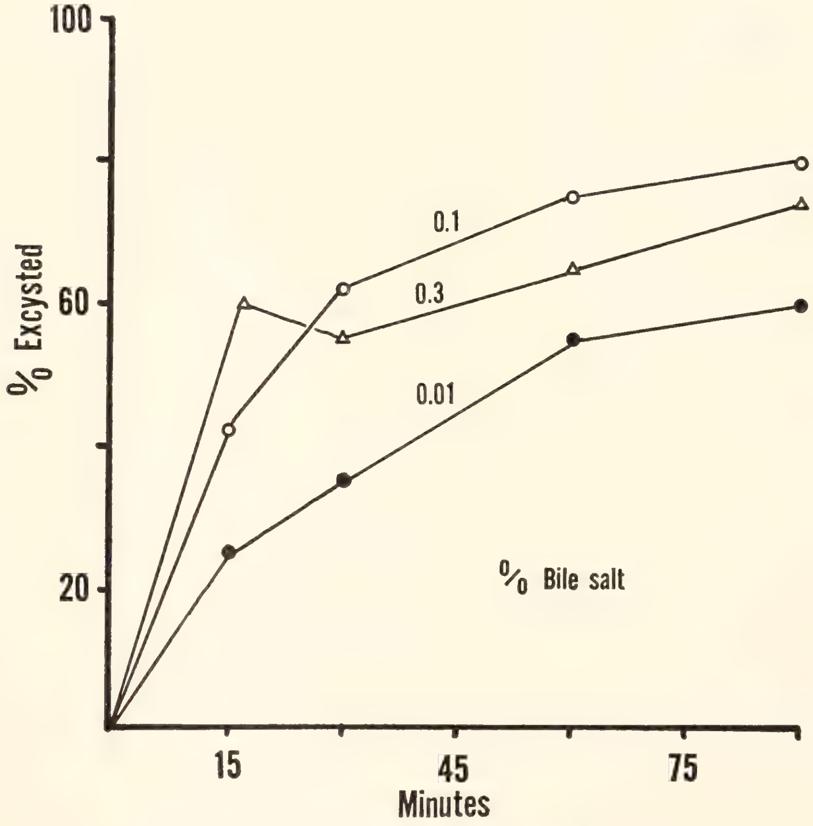

FIGURE 4. Effect of bile salt on excystment. Conditions: 30 minutes in 1% pepsin (pH 2) at 37° C., removed to 1% trypsin plus the concentration of sodium taurocholate shown (pH 7).

Sodium taurocholate was not essential for excystment, but it enhanced the effect obtained with trypsin. Its effect was related to its concentration. Initially, the

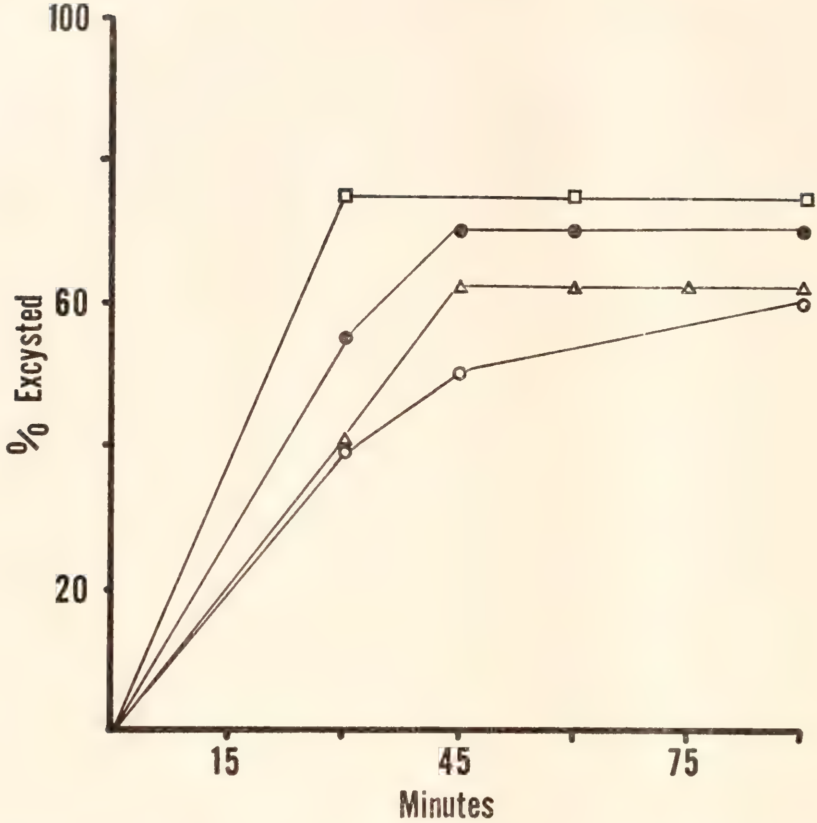

Figure 5. Effectiveness of diluted media on excystment. Representative experiments shown. Conditions: 30 minutes in 0.1% pepsin (pH 2) at 37° C., remove to; ○—○ 0.1% trypsin plus 0.005% sodium taurocholate (NaT), ●—● 0.1% trypsin plus 0.01% NaT, □--□ 0.1% trypsin plus 0.02% NaT, △—△ 0.05% trypsin plus 0.01% NaT.

rate of excystment increased with higher concentrations, but 0.3% bile salt slightly repressed the rate after 15 minutes (Fig. 4).

Optimum excystment in trypsin-bile salt after pepsin treatment was attained with 1% trypsin plus 0.1% sodium taurocholate pretreated for 30 minutes with 1% pepsin. However, excellent excystment (65–75%) occurred also with 10-fold and greater dilutions of these substances (Fig. 5).

Discussion

Stimuli necessary for excystment of the metacercariae of trematodes are found in the host's intestine. However, escape from enclosing membranes entails responses which differ among the various species. The process is apparently more closely related to that of cestodes and acanthocephalans than that of nematodes (see Rothman, 1959; Rogers, 1962; Graff and Kitzman, 1965).

Faust and Khaw (1925, 1927) found that the metacercaria of *Clonorchis* *sinensis* must be pretreated in artificial gastric juice before it will excyst in artificial intestinal juice, but Bacha (1960) observed that *Zygocotyle lunata* metacercariae will undergo excystment when placed into the caecum of the white rat, without exposure to stomach or small intestine activity.

Much of the information on the excystment of metacercariae is to be found in the attempts to free larvae of their cysts by the "artificial digest method." Stunkard (1930) observed that *C. lingua* metacercariae would excyst in distilled water or Ringer's solution after four hours in a saturated pancreatin solution. Ferguson (1940) found that the metacercariae of *Posthodiplostomum minimum* escape their cysts in an acidified solution of crude pepsin incubated at 37° C. Hunter and Chait (1952) were able to excyst the larvae of *Gynaecotyle adunca* by treating them in a pepsin-HCl solution, followed with a sea water wash. *Apatemon pellucidus* metacercariae are free of their cysts within 10 minutes in a trypsin solution after pepsin treatment (Hoffman, 1959).

The role of bile and bile salts in metacercarial excystment has received some attention. Oshima *et al.* (1958) and Oshima and Kihata (1958) showed that the excystment of *Paragonimus westermani* metacercariae took three hours without bile salts, but only 10 to 30 minutes upon addition of sodium cholate or sodium deoxycholate. The metacercaria of *Metagonimus yokagawai* does not excyst in pepsin or pancreatin unless pig bile is added (Kobayashi *et al.*, 1959). Wickerhauser (1960) showed bile and a pepsin pretreatment to be requirements for the excystment of *Fasciola hepatica* metacercariae in trypsin. However, Dawes (1961) recovered larvae of *F. hepatica* from cysts injected into the peritoneal cavity of mice and, therefore, doubts that digestive enzymes are indispensable for the excystment of this species. Dixon (1964) suggests that the process of excystment of *F. hepatica* metacercariae is an active one since digestion of the cyst wall by host enzymes does not appear to be necessary.

Larval activation is an important component of cysticercoid and cystacanth excystment (Rothman, 1959; Graff and Kitzman, 1965). None of the conditions tested were effective in activating larvae of *C. lingua*, but overt muscle activity may not be as necessary for excystment of this trematode. In its development the metacercaria enlarges faster than the cyst, and the fish host surrounds it with a strong capsule. Undoubtedly, further larval growth leads to an increasing pressure on the inside of the cyst which could be instrumental in promoting escape from weakened membranes.

No chemical or physical condition tested was an absolute requirement for excystment of *C. lingua* metacercariae. Within physiological limits, temperature and pH had no direct effect on excystment. Their effect was in the activation of hydrolytic enzyme systems which led to excystment within a biologically useful time. The pepsin solutions affected the cyst at a rate influenced by concentration, pH, and temperature. The pH's above 1 had a rapidly decreasing effect. Since mammalian and avian stomach contents usually average about pH 3.5 (Rothman, 1959; Farner, 1960), the cyst, *in vivo*, is probably not breached before entering the small intestine.

Apparently, pretreatment in pepsin is necessary for excystment in trypsin if bile is not present. Excystment did not occur in trypsin or in trypsin after treatment with Ringer-HCl. However, some excystment did occur without gastric pretreatment. Fresh gull bile was not effective, but gull intestinal juice showed greater activity than the combinations of chemicals that were tested. A synergistic effect was observed between bile salt and trypsin. Both gull bile and sodium taurocholate were effective. Increasing the concentration of bile increased the rate of excystment initially, but the higher concentrations repressed excystment upon longer exposure. Recent work with commercial bile salt preparations indicates that traces of several bile acids or salts might be present in the experiments relating to bile (Smyth and Haslewood, 1963).

Pepsin digestion followed with trypsin in conjunction with surface-active and reducing agents proved most effective in stimulating excystment over a wide range of concentrations.

Comparison of excystment in pancreatin with that in trypsin gave no indirect evidence that pancreatic lipolytic and amylolytic enzymes were active in the process.

Appreciation is expressed to Drs. C. P. Read, under whose direction this work was carried out, and F. M. Fisher, Jr., for reading the manuscript.

SUMMARY

1. The excystment of *C. lingua* metacercariae is mediated by the action of digestive enzymes. The same sequence of enzyme solutions as that met with in the host's digestive tract is required.

2. Reducing and surface-active agents, and a carbon dioxide atmosphere enhance excystment.

3. None of these factors proved to be an absolute requirement, but their combined effect was to stimulate excystment within a biologically useful time period.

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CONTRACTILE PROTEINS OF MARINE INVERTEBRATE SPERMATOZOA[1]

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One of the most striking and significant contributions to our insights into the nature of sperm motility has been the observation that "spermosin," a protein with ATP-ase activity obtained from bull spermatozoa, could enter into a reactive dual protein complex with skeletal muscle actin (Burnasheva, 1958). Employing methods conventionally used in the extraction of myosin and actomyosin, Burnasheva prepared the spermosin which undergoes a reversible combination with rabbit psoas actin. Addition of adenosinetriphosphate decreased the viscosity of the synthetic actospermosin by nearly a factor of two. This dramatic demonstration of the interaction of actin with spermosin not only appears to duplicate the molecular interaction of actin with myosin, but also suggests that the structural specificity of spermosin closely resembles that of the complementary combining sites of myosin for actin. But since, even after prolonged extraction the flagella yield a protein which by itself exhibits no viscosity change in the presence of ATP, Burnasheva concluded that the flagella must lack actin.

However, assuming that, at the molecular level, at least a formal similarity exists between the flagellar movement and muscular contraction, one may suppose that flagella may contain a second protein, analogous to actin, which, interacting with spermosin through the mediation of ATP, participates in the production of the flagellar wave. If this protein were structurally bound in such fashion as to prevent its ready extraction by the same means used to obtain spermosin, an alternative extraction procedure could perhaps release the actin analog from the flagellar organelles.

Treatment with acetone or detergents is frequently useful in freeing such proteins from cell particulates. Watery extracts of acetone powders of flagella isolated from sonicated starfish spermatozoa form a clear gel-like pellet which contains a bound nucleotide (Plowman and Nelson, 1962). Mohri (1964) adapted Gibbons' (1963) use of digitonin to solubilize bonds which apparently hinder the extraction of contractile proteins from sea urchin and fish sperm. The digitonin treatment provided the means for obtaining, almost quantitatively, a highly active ATP-ase in solution in alkaline 0.6 *M* KCl. After dialysis against dilute buffer, further extraction of this protein with cold perchloric acid yielded a substance with an adenine nucleotide ultraviolet absorption peak (258 mμ; one of the characteristics of actin is its high content of bound adenine nucleotides). Mohri evidently obtained an actomyosin-like protein from the spermatozoa.

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A. G. Szent-Györgyi had previously shown that reagents containing strongly electronegative anions, such as KI, which depolymerize actin, also readily extract the actin from muscle (1951). Since inordinately large volumes of sperm suspensions are needed to prepare the acetone powders (Plowman and Nelson, 1962) and since nonpolar solvents denature myosin, a combination of the Mohri procedure with that of Szent-Györgyi was employed in extraction of flagellar proteins for a study of their viscometric properties.

Materials and Methods

Gonads removed from the arms of mature male starfish, *Asterias forbesi*, were minced in filtered sea water and the spermatozoa poured through four layers of cheesecloth. The spermatozoa were disrupted by sonic oscillation with a Bronwill Ultrasonic Probe at a frequency of 20 kc./sec. for 45–60 seconds in an ice bath. Centrifugation at 1000 *g* twice, for 10 minutes each time, removed most of the heads. The tails could then be sedimented in 10 minutes at 10,000 *g*. The residues were suspended in five volumes of cold 0.6 *M* KI, 0.06 *M* $Na_2S_2O_3$ and 0.1% digitonin (in 2.5 m*M* Tris buffer pH 8.3 and 30 m*M* $MgCl_2$) and extracted for one hour in the refrigerator. After the suspension was centrifuged for 10 minutes at 5000 *g*, the supernatant was decanted and diluted with 15 volumes of ice-cold deionized water and the pH adjusted to 4.65 by addition of 1.0 *M* sodium acetate buffer. The precipitate settled overnight in the refrigerator; excess fluid was removed by aspiration and the precipitate concentrated in the centrifuge at 5000 *g*. Solid $NaHCO_3$ was added to neutralize the precipitate. At this point some of the preparations were dissolved in 0.6 *M* KCl and tested viscometrically for ATP-sensitivity.[2] Other preparations were dispersed in 0.6 *M* KI + 0.06 *M* $Na_2S_2O_3$ + 10^{-4} *M* ATP, using a Teflon homogenizer, and an equal volume of cold deionized water was added. Insoluble material was removed by centrifugation and to the supernatant was added one quarter volume of 95% ethyl alcohol chilled to −20° C. The mixture was then dialyzed against 6.7 m*M* phosphate buffer, pH 7, containing 10 μM ATP, with changes every half hour for four hours; the precipitate was removed and to each 100 ml. of dialysate were added 2 ml. of saturated KCl. This was kept at room temperature for one hour, chilled and then centrifuged in a Beckman-Spinco Model L Ultra-centrifuge #40 rotor for 60 minutes at 35,000 R.P.M. The supernatant was carefully drained off and the clear colorless pellets were dissolved in 2 ml. of 10 μM ATP, 10 μM ascorbic acid. These solutions were tested in Ostwald viscometers in a constant temperature bath at 0° C. Each sample was run through the viscometer at least 5 times, usually at four-minute intervals. Protein concentrations were determined according to the method of Lowry *et al.* (1951).

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[2] On the basis of its viscometric response to the addition of ATP, the product at this stage of preparation is referred to as flactospermosin. Subsequent to its treatment with the 95% alcohol and removal of the spermosin, the material is called flactin.

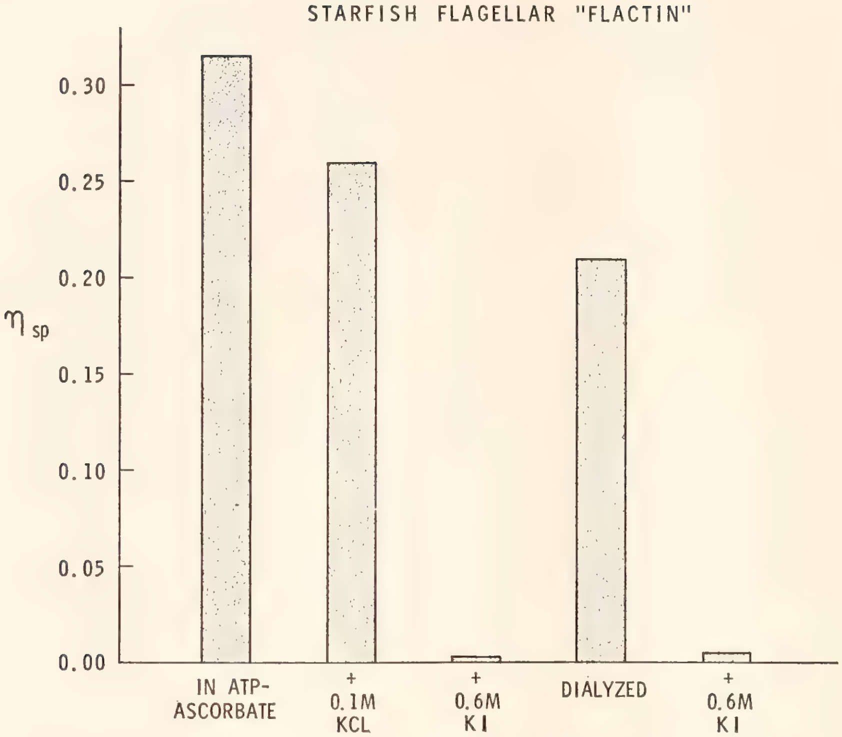

FIGURE 1. Starfish flagellar flactin. Ordinate, specific viscosity of flactin. Two-ml. samples at 0° C. Initial sample prepared from flagellar extract after removal of KI by dialysis against ATP-ascorbate, polymerization in 0.1 M KCl and centrifugation at 100,000 g to form clear pellets. Pellets dissolved in 10 μM ATP-ascorbate; protein concentration 4 mg./ml. Second bar, addition of 0.2 ml., 1 M KCl; no further polymerization. Third bar, addition of 0.2 ml. of 6 M KI + 0.6 M $Na_2S_2O_3$; depolymerization. Fourth bar, removal of KI by dialysis against ATP-ascorbate; repolymerized. Fifth bar, depolymerization by addition of $\frac{1}{10}$ volume of 6.0 M KI + 0.6 M $Na_2S_2O_3$.

RESULTS[3]

Viscosity measurements made on the redissolved flagellar "actin" pellets gave outflow times ranging from 125 to 135 seconds, depending on the protein concentration, while additions of KI (final concentration of 0.6 M) lowered the time to about 105–110 seconds.

The outflow time of pure solvent (0.6 M KI + 0.06 M $Na_2S_2O_3$) was 103 seconds and of the ATP-ascorbate (10 μM) was 107 seconds.

In the first experiment (see Fig. 1) the specific viscosities were determined for a sample of the starfish sperm flagellar "actin" which had a protein content of 4–5 mg./ml. Addition of KCl (final concentration = 0.1 M) did not cause any apparent further polymerization; in fact the outflow time decreased slightly. However, 0.2 ml. of 6 *M* KI + 0.6 *M* $Na_2S_2O_3$ added to the 2-ml. sample caused up to a 25% decrease in outflow time. The specific viscosities had dropped from 0.315 to 0.260 (KCl) to 0.04 (KI), pointing to a possible depolymerization and presumably conversion from a fibrous to a globular form.

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[3] The viscosity terms used in this paper are defined as follows:

Specific viscosity (η_{sp}) = relative viscosity −1, where relative viscosity is viscosity of the protein solution divided by solvent viscosity.

Reduced viscosity (η_{sp}/c) = specific viscosity divided by the protein concentration.

Intrinsic viscosity = the limit of (η_{sp}/c) as c approaches zero.

When the samples retrieved from the viscometers were dialyzed overnight against cold 10 μM ATP in 6.7 m*M* phosphate buffer pH 7, the flagellar "actin" or "flactin" apparently reverted to the more fibrous form. This brought the outflow time back up to about 126 seconds and a specific viscosity of 0.21. Addition of 1/10 volume of 6*M* KI + 0.6 *M* $Na_2S_2O_3$ again caused an apparent depolymerization, dropping the outflow time to 106 seconds and the specific viscosity to 0.03.

In other samples of the flactin with slightly lower protein content, the KI caused a fairly rapid initial depolymerization followed by a slower small additional decrease in viscosity. Here again, dialysis against the ATP-phosphate buffer restored the viscosity almost to the initial value.

A rough estimate of the intrinsic viscosity of the flactin may be calculated by dividing the specific viscosity of the depolymerized flactin by its concentration. The specific viscosity of the sample in Figure 1 after treatment with KI was 0.04 and the concentration was about 0.4 g./100 ml., giving about 0.1 for the intrinsic viscosity. According to Tsao (1953), who was studying skeletal muscle actin, this reflects the condition of a solution containing particles of considerable asymmetry. Several determinations of reduced viscosity of a flactin preparation (ordi-

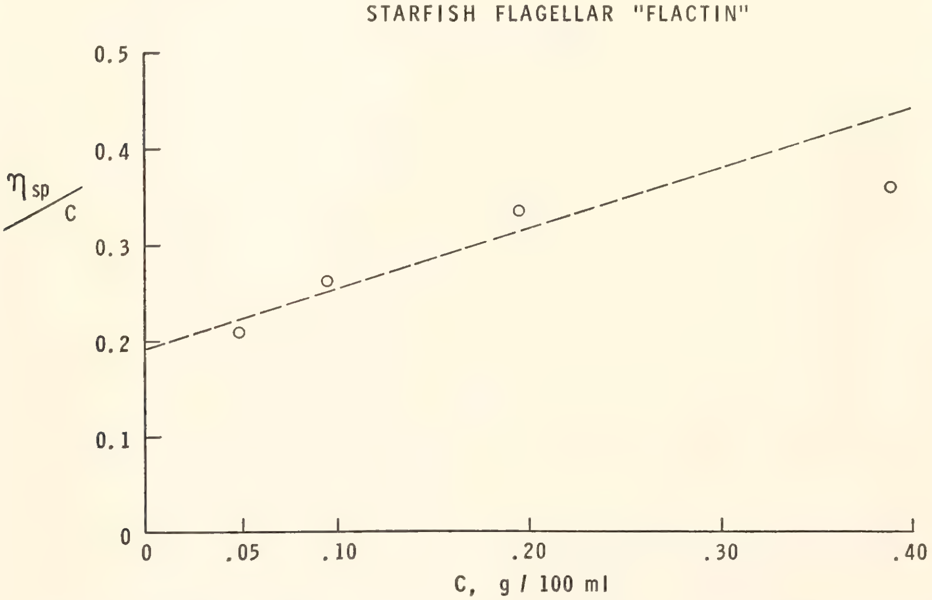

FIGURE 2. Starfish flagellar flactin. Intrinsic viscosity. Ordinate: Reduced viscosity; abscissa: concentration of flactin in g./100 ml. Samples in 10 μM ATP-ascorbate. Extrapolation to zero protein concentration gives value of about 0.2, indicating fair degree of asymmetry of flactin. Viscosity measured at 0° C.

nates: η_{sp}/C) at different concentrations of the sample dissolved in 10 μM ATP-ascorbate solution provide the data for the graph (Fig. 2). Protein concentrations, in g./100 ml., appear on the abscissa. Extrapolation of the curve to infinite dilution of the protein gives an intrinsic viscosity of about 0.2.

Samples dissolved in 0.6 M KCl prior to the step at which spermosin was removed by denaturation in cold alcohol were tested for sensitivity to ATP. This material, referred to as "flactospermosin," had ATP-ase activity and underwent precipitation after 10-fold dilution with 0.1 mM $MgCl_2$ in the absence of ATP and superprecipitation on dilution in the presence of 0.2 mM ATP.

Two aliquots of flactospermosin in 0.6 M KCl had outflow times in the Ostwald viscometers of between three and four minutes. Addition of 1/10 volume of 2 mM ATP-ascorbate solutions caused rapid drops in the outflow time followed by slower

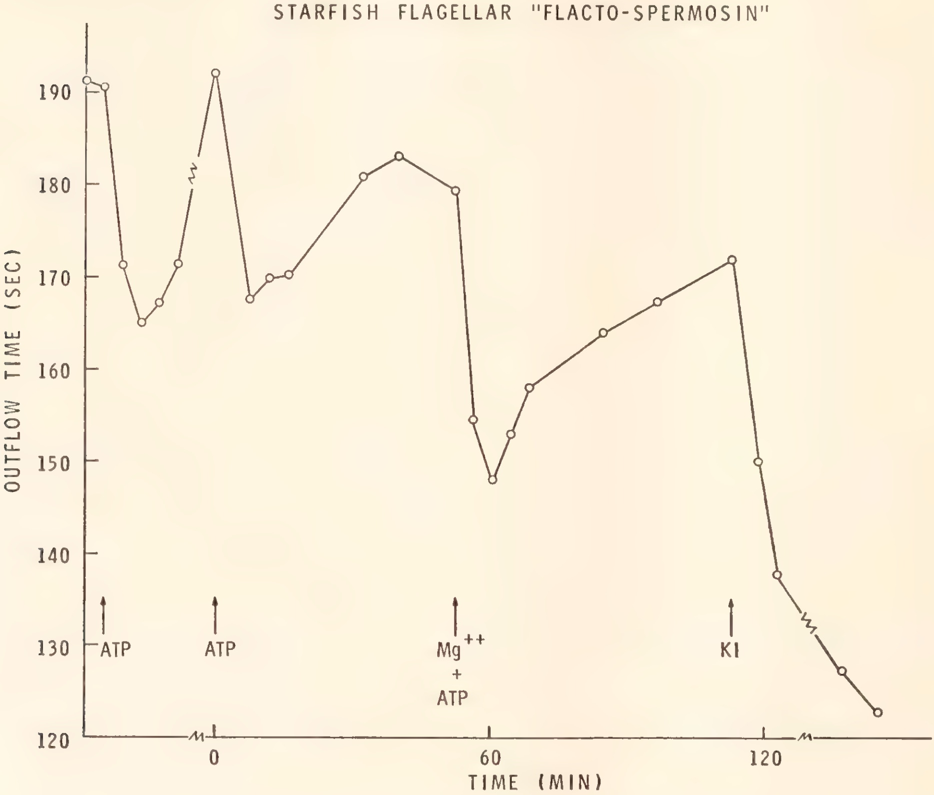

FIGURE 3. Starfish flagellar flactospermosin. Effect of ATP on viscosity of 0.6 M KCl solutions of flactospermosin. Two-ml. samples of flactospermosin; $\frac{1}{10}$ volume of 2 mM ATP added; $\frac{1}{10}$ volume of 4 mM $MgCl_2$ added at arrow. Ordinate: outflow time in Ostwald viscometer. Abscissa: duration of experiment in minutes to indicate relative time required for "contraction" and "relaxation" showing ATP-sensitivity of flactospermosin. In terms of specific viscosity, deviations from the initial value of 0.83 subsequent to additions of the various reagents and recoveries resulted in the following minima and maxima: 0.57, 0.84, 0.59, 0.74, 0.41, 0.64 and finally 0.17.

recovery to, or nearly to, the initial viscosity. Figure 3 graphically illustrates the course of one of these experiments. The ordinate shows the outflow time in seconds, in the Ostwald viscometer, at 0° C. The abscissa represents the time scale with readings usually at four-minute intervals and also shows the relative amount of time required for the effect of ATP addition and recovery. (The breaks in the abscissa, however, indicate two-hour intervals.) Addition of 0.4 mM $MgCl_2$ along with the ATP caused a somewhat greater drop in viscosity, followed by a delayed and less complete reversion to the initial viscosity. This may be due to a slight diminution of the ATP-ase activity of the system caused by the Mg^{++}, since the viscosity drop, which could be attributable to dissociation of the flactin-spermosin complex, depends on the presence of unhydrolyzed ATP, and 1 mM $MgCl_2$ inhibits the ATP-ase activity of muscle proteins under these conditions. Finally, 0.6 M KI appeared to disrupt the interaction of the dual proteins in this complex, probably through depolymerization of the flactin, which completely prevented the recovery process.

Discussion

While this is intended, not as definitive, but primarily as a progress report, the results appear sufficiently interesting to merit presentation at this juncture.

Burnasheva established that mammalian spermatozoa contain a "contractile" protein, spermosin, which, possessing ATP-ase activity and combining reversibly with muscle actin, physiologically resembles muscle myosin in these respects. Spermatozoa thus may avail themselves of the potential for utilizing interaction between two proteins for producing and propagating a flagellar wave. So far, some of the positive evidence adduced for the existence of an actin-like protein in mammalian sperm has been based on immunocytochemical studies (Nelson, 1962; Nelson and Plowman, 1963). This work, currently being reinvestigated, seems to be substantiated (Young, personal communication). However, recently published reports suggest that fowl actin may not be antigenic in rabbits but that "so-called antiactin antisera are directed against native and classical tropomyosin and perhaps other proteins involved in contraction and relaxation of artificial actomyosin" (Cahn, 1965). On the other hand, Pepe (1966), using chicken breast muscle, prepared what purports to be an anti-actin distinct from anti-tropomyosin, for immunocytochemical studies on chicken myofibrils. In our laboratory, rat actin and rat myosin served as antigens in immunocytochemical analyses of rat epididymal sperm.

The bulk studies on the actin-like component of the contractile system of the sperm have mainly centered on the starfish material because of its availability in greater quantities than either rat or bull sperm, although currently such studies on the mammalian material are in progress.

One of the complicating features in the investigation of actin properties, referred to above, has been the variable degree of contamination with tropomyosin. Special precautions and modifications in the conventional acetone methods for extraction and purification of the actin may reduce this to a minimum (Carsten and Mommaerts, 1963; Baranyi, Baranyi and Guba, 1957). In the present study, because these methods inactivate the ATP-ase, no effort has been made to avert this problem, but while no evidence concerning the possible occurrence of a tropomyosin-like substance in sperm flagella has come to light, a preliminary diagnostic check by the method of Szent-Györgyi and Kaminer (1963) indicates the strong possibility for the existence in very small amounts of a "tropospermosin" (Kaminer, personal communication). While this in itself is an interesting development and of course opens up new lines for investigation on sperm contractile proteins, nevertheless, it need not detract from the material at hand. In any event, flactin does not hydrolyze ATP, and the experiments illustrated in Figure 3 depend on retention of ATP-ase activity by the flactospermosin.

As previously reported (Plowman and Nelson, 1962), the flactin showed a predisposition to salt out when the pellets were dissolved in media of ionic strengths above 0.05. Presumably aggregation accompanies polymerization as the KCl concentration approaches 0.1 *M*. Attempts to determine sedimentation constants in the analytical ultracentrifuge reflected the tendency of the flactin to aggregate; a fairly sharp peak would form and disappear rapidly before the centrifuge attained a speed of 25,000 R.P.M. This phenomenon will be examined in detail. In ATP-ascorbate (10 μM) or following dialysis against the 6.7 mM phosphate buffer + 10 μM ATP employed to repolymerize the flactin after depolymerization in KI, the solutions were slightly opalescent. During viscometry, the flactin would flow freely and reproducibly, to within fractions of a second, without indication of particle formation.

Intrinsic viscosity, determined following dilution of individual flactin preparations to decreasing protein concentration in ATP-ascorbate, calculated out to 0.2 by extrapolation to zero concentration. This closely approximates that obtained by Tsao (1953) for the monomeric form and is a little less than half his value for a postulated dimeric form, of skeletal muscle actin. Tsao further, allowing for a contribution of hydration to the asymmetry factor (0.39 g. H_2O/g. protein) and assuming a partial specific volume of 0.74, concluded that the actin monomer has an axial ratio of about 12, as a prolate ellipsoid of revolution.

The experimental results so far indicate that sperm flagella contain a second protein (spermosin being the first), associated with configurational changes in molecules attendant upon the formation of the propulsive undulatory wave. This protein, flactin, appears to undergo polymerization readily, to interact reversibly with spermosin, may be asymmetrical in shape, and lacks the ability to split ATP. Other evidence which may support these conclusions lies in the dilution-precipitation behavior of the flactospermosin extracts and the effects of dilution on sea water or isotonic KCl suspensions of isolated sperm tails. In the first instance, dilution-precipitation followed by super-precipitation in the presence of ATP characterizes actomyosin sols. Suspensions of *Mytilus* sperm tails also react in this fashion (Nelson, 1959), but addition of ATP markedly delays the precipitation of diluted starfish sperm tail suspensions, as if the ATP were serving as a relaxing agent. However, to the contrary, ATP expedites the precipitation of the diluted starfish sperm extract, flactospermosin. Speculation on the basis of this apparent discrepant behavior would probably be premature although we have observed some suggestive differences in the motility characteristics of the sperm of *Mytilus*, *Asterias* and other marine invertebrate species.

While these findings point to a possible formal similarity between sperm tail bending and muscle shortening at the level of protein-protein interaction, ATP and divalent cation shifts, no attempt should be made to impose morphological identity on the respective fibrous elements concerned. At the fine structure level, one need not invoke a sliding filament mechanism for flagellar action, even though Satir (1964) reasons that sliding filaments must serve as the basis for ciliary action since he could see no fibers shorter than others in electron micrographs of lamellibranch gill cilia. The immunocytochemical studies show that flactin comprises the cortical region of the coarse longitudinal fibers while the spermosin, in intimate contact with the flactin, occupies the core portion of these same fibers in rat sperm (Nelson and Plowman, 1962). Since only about 3% shortening of the fibers will produce the radius of curvature of the sinusoidal flagellar wave, the necessary configurational change could accompany the interaction of flactin and spermosin in the presence of ATP.

SUMMARY

1. Starfish sperm flagella prepared by ultrasonic disruption were extracted with digitonin and 0.6 *M* KI.

2. Flactin, a flagellar protein analogous to muscle actin, was obtained which undergoes viscosity changes similar to depolymerization and polymerization. Like actin, flactin does not hydrolyze ATP.

3. Flactospermosin, however, like actomyosin, does split ATP, and also precipitates when diluted 5–10-fold with deionized water. If ATP is present, superprecipitation occurs. In 0.6 *M* KCl solutions of flactospermosin, ATP causes a rapid drop in viscosity, which reverts to the initial value as the ATP becomes hydrolyzed.

4. The intrinsic viscosity, reduced viscosity extrapolated to infinite protein dilution, of flactin has a value of about 0.2, indicating that this is a fairly asymmetric molecule.

5. While spermatozoa appear to possess the potential for employing a dual protein complex in flagellar wave production, this does not imply that, on a fine structural level, a sliding filament mechanism underlies flagellar movement. Upon interaction of the flactin and the spermosin, ATP hydrolysis could induce the necessary configurational change.

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FOOD, REPRODUCTION AND ORGANIC CONSTITUTION OF THE COMMON ANTARCTIC ECHINOID STERECHINUS NEUMAYERI (MEISSNER)[1]

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Studies have been made on the reproduction and nutrition of regular echinoids of California (Lasker and Giese, 1954; Bennett and Giese, 1955; Giese *et al.*, 1958; Boolootian *et al.*, 1959; Giese, 1959, 1961; Holland and Giese, 1965). The general findings have been in accord with similar studies on other echinoids of temperate and tropical regions (Hyman, 1955, pp. 461–506; Harvey, 1956; Nichols, 1964; Giese *et al.*, 1964). The biology of polar echinoids, which live in an environment where light and phytoproduction are markedly seasonal and temperature is constantly near freezing, has remained virtually unknown, largely because of the difficult working conditions. The establishment of a permanent biological laboratory at McMurdo Station, Antarctica (79°S., 166°E.) (Wohlschlag, 1963) has made possible the work on a polar sea urchin, *Sterechinus neumayeri*, presented in this paper.

Sterechinus neumayeri (Meissner), one of the regular echinoids (Echinidae), is among the most common animals in shallow water of McMurdo Sound (Dearborn, 1965). However, because McMurdo Sound is covered with sea ice most of the year, specimens of *S. neumayeri* are difficult to collect. Only 37 specimens of *S. neumayeri* were obtained for this study from McMurdo Sound during samplings throughout 1961. Although this sample is small it proved adequate to provide some basic information on the food, reproductive periodicities and organic constitution of this echinoid, which is presented here for comparison with work previously done on echinoids of temperate and tropical regions.

Materials and Methods

The specimens of *S. neumayeri* used for this study were collected from Cape Royds (77°33′S., 166°07′E.), Cape Evans (77°38′S., 166°24′E.) and Turtle Rock (77°45′S., 166°46′E.) in McMurdo Sound off the southwestern coast of Ross Island, Antarctica. These collecting localities have been previously described

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(Pearse, 1965). The number of animals collected at each locality and the date of each collection are given in Table II. Most of the specimens were taken with a dipnet from 1 to 3 m. depth, either through cracks in the sea ice, or while wading, or from a small pneumatic raft when the sea was free of sea ice cover (January to mid-April). Specimens of *S. neumayeri* were difficult to collect between mid-April and December when the sea ice solidly covered the southern part of McMurdo Sound. However, on November 11 one animal was taken by dipnet from the leading edge of the sea ice cover at Cape Royds and on December 7 another animal was snared in a trap set through a hole cut in the sea ice at Cape Royds and a third was similarly captured at Cape Evans.

The animals were transported in insulated gallon jugs to the nearby biological laboratory at McMurdo Station within a few hours of their collection. Wet animal weight and gonad weight were taken, and a gonad index for each specimen was calculated by: gonad weight times 100 divided by animal wet weight. Sexes were distinguished by microscopic examination of gonadal smears. Food pellets in the gut and fecal pellets were squashed and inspected microscopically.

TABLE I

*Comparison of organic constitution in frozen and trichloroacetic acid (TCA) treated gonadal tissue of S. neumayeri in per cent per unit dry weight**

	Lipid	Carbohydrate	Lowry protein	Kjeldahl protein	Total nitrogen
Testes					
frozen	19.5 ± 1.8	14.1 ± 1.4	31.0 ± 9.0	21.2 ± 2.5	8.5 ± 0.7
TCA	20.0 ± 1.6	13.7 ± 0.9	33.8 ± 6.3	15.8 ± 3.9	8.2 ± 0.6
Ovaries					
frozen	26.1 ± 3.0	16.3 ± 3.8	32.0 ± 7.3	16.4 ± 3.0	6.7 ± 0.3
TCA	26.2 ± 2.6	15.5 ± 2.9	30.5 ± 6.3	12.6 ± 0.5	6.8 ± 0.4

* The sample consisted of 5 male and 5 female specimens collected on April 5, 1961, from Cape Evans, McMurdo Sound, Antarctica. Mean values are given with one standard deviation.

Small pieces of gonadal tissue were taken from each specimen and fixed in alcoholic Bouin's solution for 48 hours. These samples were dehydrated with acetone, cleared with benzene, and embedded and stored in paraffin. Sections ($5\ \mu$) were stained by Mallory's triple technique. The diameters of 50 oocytes, each with the nucleolus showing in section, were measured for each ovary sample, and the frequency of occurrence of the different-sized oocytes plotted as has been done for the asteroid *Odontaster validus* (Pearse, 1965).

For biochemical analyses, small pieces of the gonads, the ambulacral and interambulacral test areas at the ambitus with spines, the gut from the esophagus to the rectum, washed free of contents, and the gut contents were placed in small dishes and "fixed" with a few drops of 5% trichloroacetic acid. The tissue samples were dried either *in vacuo* over concentrated H_2SO_4 or in a closed chamber with air circulated continuously over the tissues and NaOH flakes. A few duplicate gonadal tissue samples were also frozen at − 70° C. and lyophilized. The samples were stored in sealed No. 2 tin cans with anhydrous $CaCl_2$ and shipped to Stanford University for biochemical analyses.

Total nitrogen in the tissues was determined by the Kjeldahl method (Markham, 1942). The protein in an aliquot of each tissue sample was precipitated in boiling 10% trichloroacetic acid and filtered. The nitrogen values (the non-protein nitrogen) in the filtrates determined by the Kjeldahl method were subtracted from the total nitrogen values obtained by assaying a piece of tissue to give estimates of the protein nitrogen values. Multiplication of the protein nitrogen values by the conversion factor 6.25 gave "Kjeldahl protein" values. Protein estimations were also made by the Lowry method (Lowry *et al.*, 1951) after samples of the dried tissues were dissolved overnight in 1 N KOH, bovine serum albumin being used as a standard. Protein values estimated by this method are referred to as "Lowry Protein."

TABLE II

*Animal sizes (gm.), gonad indices and organic constitution of the gonads (% dry weight) of S. neumayeri collected from McMurdo Sound, Antarctica during 1961**

	Wet animal weight	Gonad index	Lipid	Carbohydrate	Lowry protein	Total nitrogen
Cape Evans						
February 7						
males (3)	60.0±23.4	10.1± 3.8	23.9± 2.4	12.2± 1.4	32.9± 0.5	8.1±0.6
females (2)	33.0†	14.9†	23.5, 30.5	12.2, 14.9	36.3, 30.2	7.4, 6.4
April 5						
males (5)	44.4±20.8	24.9± 3.6	20.0± 1.6	13.7± 0.9	33.8± 6.3	8.2±0.6
females (5)	55.4±11.5	31.1± 4.5	26.2± 2.6	15.5± 2.9	30.5± 6.3	6.8±0.4
April 20						
males (2)	76.9, 33.3	37.1, 22.8	21.5, 22.1	19.1, 16.6	46.6, 31.8	—
female (1)	127.5	32.4	26.2	17.3	39.9	7.1
April 28						
females (2)	36.9†	38.8†	28.6, 30.7	13.3, 16.1	35.9, 32.5	6.8, 6.6
May 5						
males (2)	77.5, 38.1	34.7, 26.5	24.0, 16.7	13.9, 18.8	34.5, 31.8	7.8, 8.5
female (1)	42.0	39.9	24.2	13.4	31.5	6.9
December 7						
unsexable (1)	85.1	27.3	39.4	10.0	41.1	6.3
November 11						
female (1)	91.4	27.6	22.4	13.8	33.3	5.9
December 7						
female (1)	30.5	10.6	28.1	13.0	32.8	6.9
Turtle Rock						
December 6						
males (6)	17.3± 4.0	4.1± 2.2	16.7± 5.7	4.9± 1.9	33.5± 5.9	8.9±1.2
females (5)	20.6± 4.8	6.6± 1.6	30.3± 5.7	4.9± 1.2	33.4± 4.3	7.7±0.8

* The tissues were fixed with trichloroacetic acid. Individual values are given for samples of one or two animals while mean values with one standard deviation are given in parentheses.

† One animal accidentally crushed before being weighed.

Lipid level in the tissues was determined by weight of the material extracted by the chloroform-methanol method (Freeman *et al.*, 1957). For carbohydrate extraction, the tissues were boiled for 10 minutes in 5% trichloroacetic acid and centrifuged. Carbohydrate in the supernatant of these extracts was estimated colorimetrically by the phenol method of Dubois *et al.* (1956), using D-glucose as the standard.

RESULTS

General habits and food

S. neumayeri occurred abundantly on basaltic gravel and rocks in water less than 15 m. in depth. The urchins were often seen with pebbles and bits of debris covering their tests. In the fall (April), when the air temperature was falling, anchor ice formed on much of the shallow bottom areas, and the urchins were often seen on this ice. Because some individuals were found preserved in perfect condition frozen into sea ice over 20 m. of water (Pearse, 1962a), these urchins probably are trapped occasionally in rising masses of anchor ice. Other large species of marine animals commonly found in association with *S. neumayeri* include the nemertean *Lineus corrugatus*, the gastropod *Neobuccinum eatoni*, the isopod *Glyptonotus antarcticus*, the asteroid *Odontaster validus* and the fish *Trematomus bernacchii.*

The average size of *S. neumayeri* collected from Turtle Rock was smaller than that of any others collected (Table II). These urchins, however, were the largest that could be taken by dipnet, and the population of *S. neumayeri* at Turtle Rock consisted of decidedly smaller animals than at either Cape Evans or Cape Royds.

At Cape Evans and Cape Royds large clumps of leafy red algae (*Iridaea* sp.) occurred frequently and may provide some food for *S. neumayeri.* However, between early February and April extensive carpets of diatoms grew on the shallow sea floor at Cape Evans. During this period the food of *S. neumayeri* consisted mainly of diatoms, the most conspicuous constituent of the food pellets in the gut. The fecal pellets from urchins collected at Cape Royds and Cape Evans in November and December also consisted mostly of diatom remains.

Turtle Rock was the site of a large rookery for Weddell seals (*Leptonychotes weddelli*), and in December, 1961, the sea floor in this area was littered with deposits of seal feces around which *S. neumayeri* aggregated. The gut of these specimens was filled with seal feces and contained relatively few diatoms.

Reproduction

The gonad indices of the samples taken from Cape Evans increased between early February and early May, and the specimens from the December 6 sample from Turtle Rock had the lowest gonad index recorded (Fig. 1, Table II). This change suggests that the gonads ripen and grow during the austral summer months and shrink following spawning during the winter or spring. However, as Holland and Giese (1965) found with *Strongylocentrotus purpuratus*, interpretation of echinoid gonad indices is sometimes difficult because of the large amounts of nutritive phagocytes in the gonads. The gonads of *S. neumayeri* also contain large numbers of these cells which are highly vacuolated (Figs. 2 and 3). Moreover, the single specimen of *S. neumayeri* of the December 7 Cape Evans sample had a high gonad index (27.3), but the gonads contained only a few gonial cells and great quantities of nutritive phagocytes. An increase in the gonad index therefore may reflect an increase of feeding with accumulation of food reserves in the nutritive phagocytes, possibly increasing the numbers of these cells, rather than an increase in gametogenic activity.

Histological sections of the gonads of *S. neumayeri* showed that oocytes of many sizes and spermatogenic cells in various stages, including spermatozoa, occurred in most of the samples (Figs. 2 and 3). The ovaries of the single female collected from Cape Royds on November 11 contained large numbers of ova ready for spawning (Fig. 2D), but there were also a few ova in the ovaries of one of the 5 females collected on April 5 from Cape Evans (Fig. 2B). Most of the testes contained spermatozoa. However, four of the 6 males collected on December 6 from Turtle Rock were spawned out (Fig. 3F), and the other two had very small gonad indices although still retaining many spermatozoa (Fig. 3E). Many of the testicular sections showed regions with little or no spermatogenic activity next to areas of intense activity (Figs. 3B, C), which is unusual for echinoid testes. In both the ovaries and testes large numbers of nutritive phagocytes were always present. Because of the presence of gametes in different stages of development in almost all of the samples, and because of the large numbers of nutritive phagocytes, reproductive periodicities in *S. neumayeri* could not be delineated by casual inspection of the histological sections.

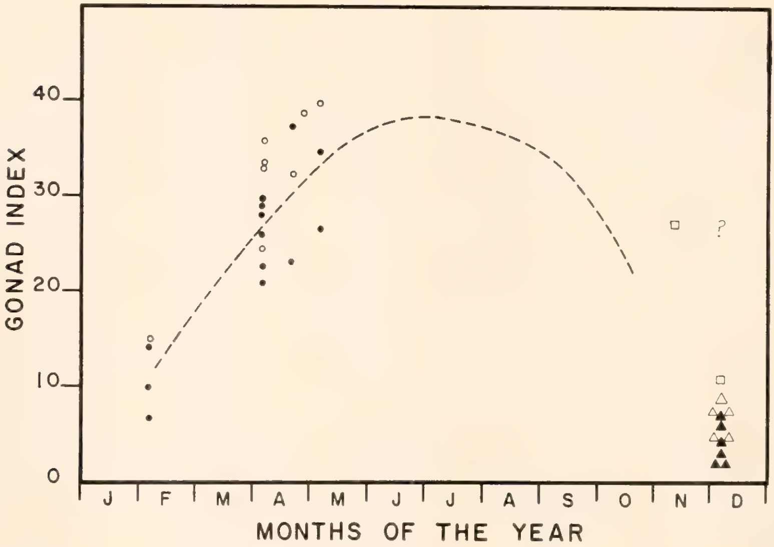

FIGURE 1. The gonad indices of *S. neumayeri* collected from McMurdo Sound during 1961. Samples came from Cape Evans (circles), Cape Royds (squares) and Turtle Rock (triangles). Solid symbols indicate males, open symbols indicate females and the "?" indicates a specimen of uncertain sex. The dotted line suggests possible changes in the gonad indices during the year.

The change in frequency of oocytes of various sizes in the ovaries was much more revealing as to reproductive periodicities in *S. neumayeri*. Frequencies with

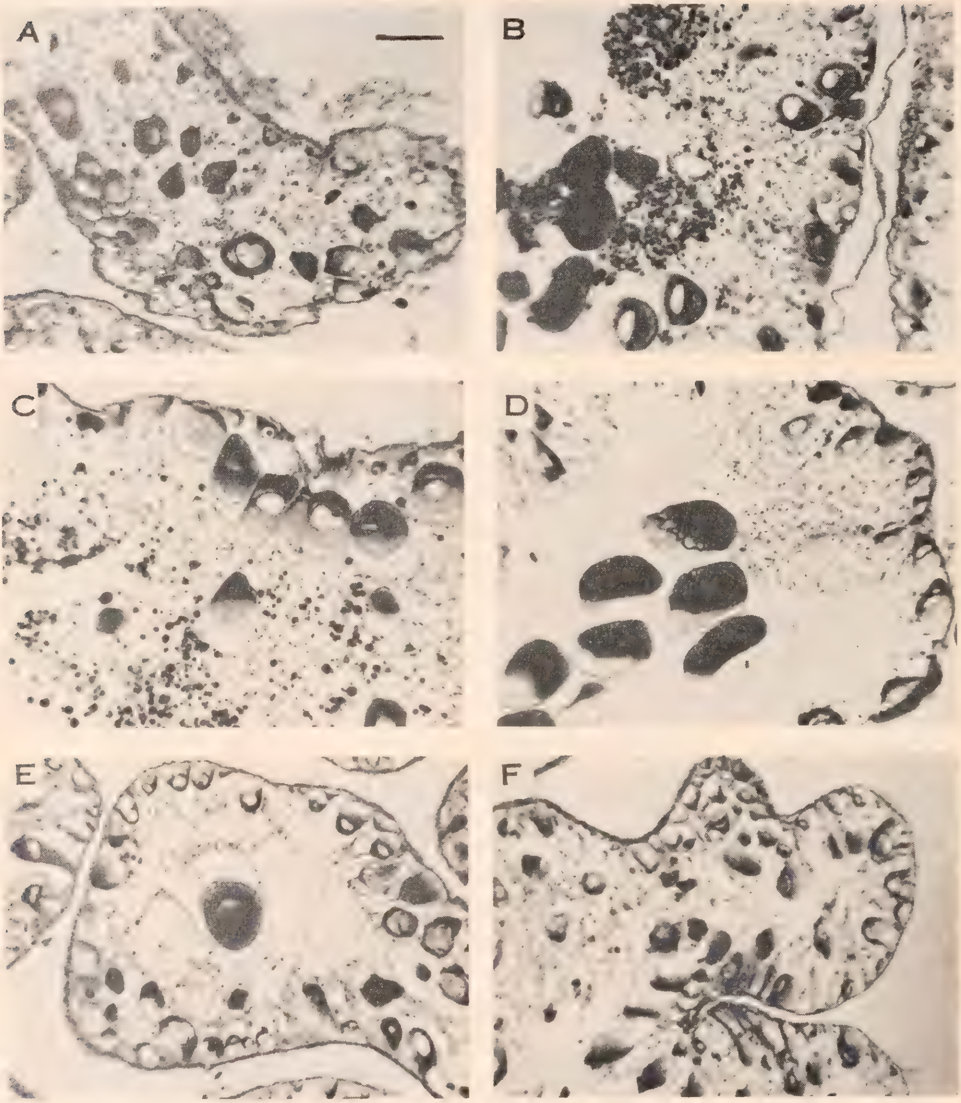

FIGURE 2. Histologic sections of ovaries from *S. neumayeri* collected at McMurdo Sound on February 7 (A), April 5 (B), May 5 (C), November 11 (D), December 7 (E), and December 6 (F), 1961. Samples for A–C were collected from Cape Evans, those for D and E were collected from Cape Royds, and the sample for F was collected from Turtle Rock. In all of the sections oocytes of various sizes occur scattered in the abundant vesicular nutritive phagocytic tissue, and ova are in the sections from the April and November samples. The scale line in A indicates 100 μ; A–F are shown at the same magnification.

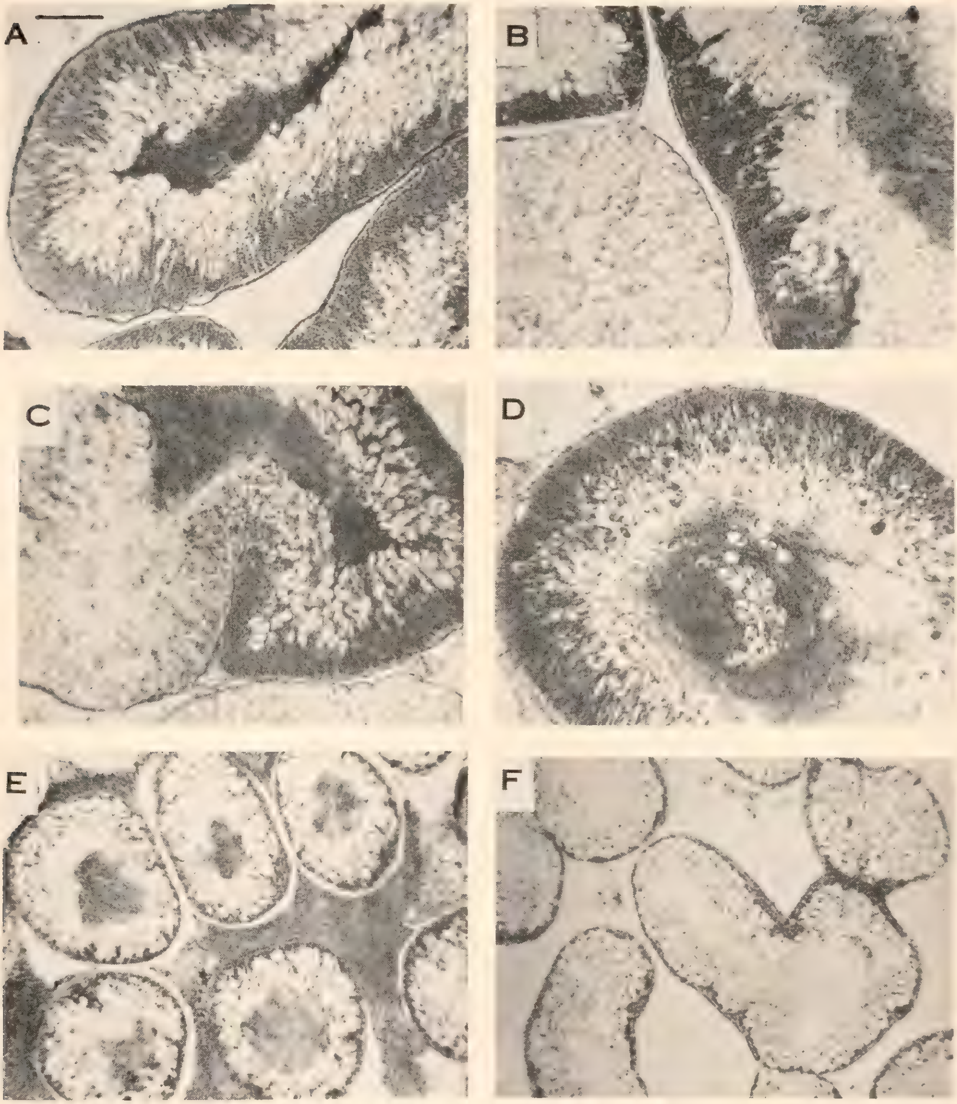

FIGURE 3. Histologic sections of testes from *S. neumayeri* collected at McMurdo Sound on February 7 (A), April 5 (B), April 20 (C), May 5 (D), and December 6 (E and F), 1961. Samples for A–D were collected from Cape Evans, and those for E and F were collected from Turtle Rock. Between February and May spermatogenesis was active among the abundant vesicular nutritive phagocytic tissue, and spermatozoa accumulated in the lumen, although as shown in B and C, spermatogenesis was not uniform along the germinal epithelium. In December the small testicular lobes contained little phagocytic tissue and spermatogenic activity was low, but the testes of some animals (*e.g.*, E) contained many spermatozoa. The scale line in A indicates 100 μ; A–F are shown at the same magnification.

which the oocytes of different sizes occur are shown in Figure 4 for all the ovary samples collected. The percentage of oocytes of any size was rather constant among individuals collected at the same time. In all the samples, except some of those from Turtle Rock, there was a clear demarcation which suggests an overlap

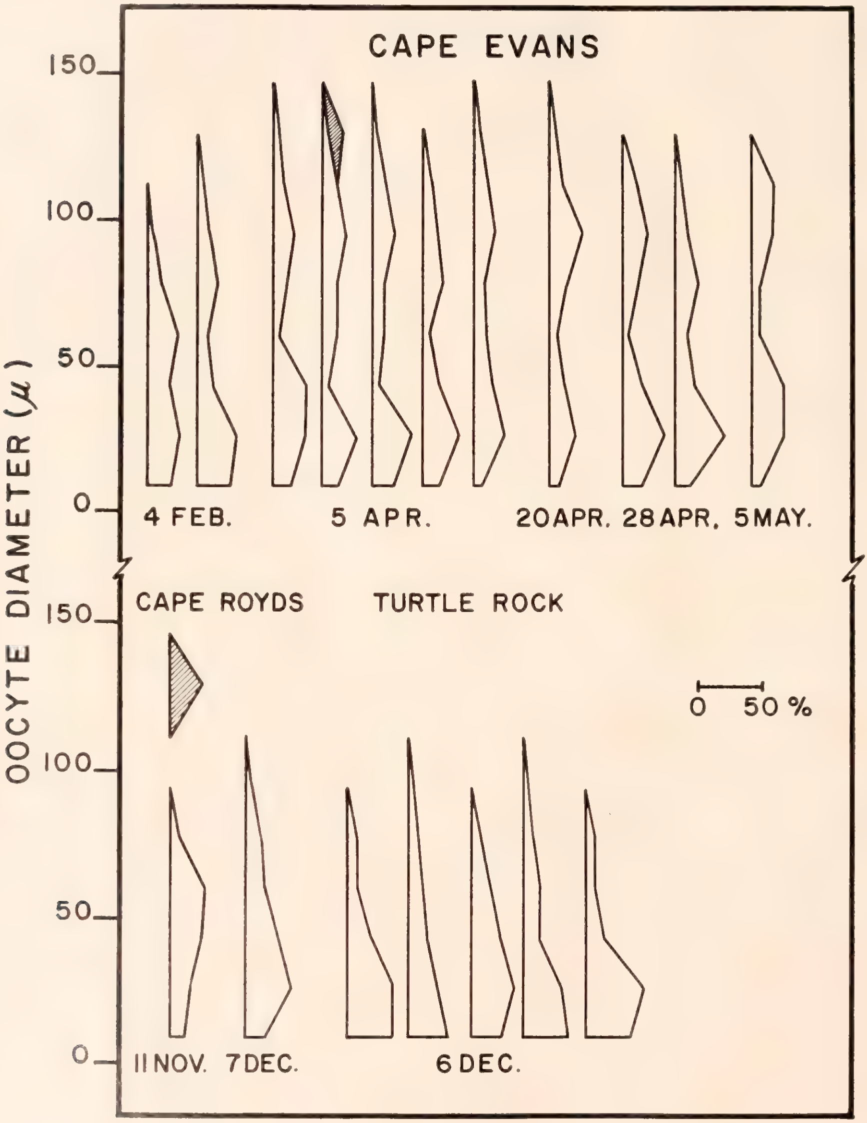

FIGURE 4. Polygons showing the frequencies (see scale) of different-sized oocytes in the ovaries of all the female specimens of *S. neumayeri* collected from McMurdo Sound during 1961. Hatched areas of the polygons represent ova; unhatched areas of the polygons represent primary oocytes.

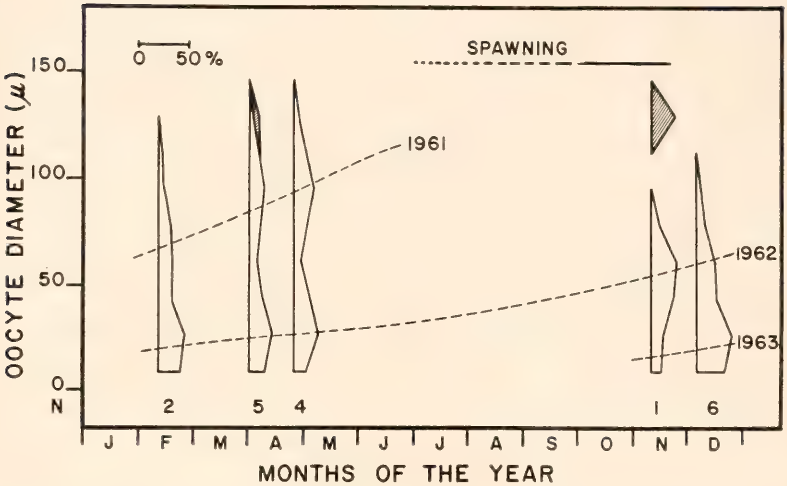

FIGURE 5. Polygons showing the oocyte frequencies (see scale) of different-sized oocytes in the ovaries of *S. neumayeri* averaged from samples collected from McMurdo Sound at different periods during 1961. The number of females averaged into each polygon is given as N. Hatched areas of the polygons represent ova; unhatched areas of the polygons represent primary oocytes. The dotted line suggests the rate of oocyte growth; "1961," "1962" and "1963" indicate the year the ova of each oocyte generation would presumably have spawned.

in two generations of oocytes, the small oocytes growing to maturity and being spawned a year after the spawning of the larger oocytes.

When the oocyte size frequencies of individuals collected during the same period were averaged and plotted through the year, a definite reproductive pattern became evident (Fig. 5). Very small oocytes appeared and began growth between November and May. As indicated in Figure 5, these young oocytes would mature into ova some 16 to 18 months after they began growth. A few ova were in the ovaries in April, and by November all the oocytes which had begun growth 18 to 24 months previously had matured into ova. Ova are therefore present in the ovaries between April and December; this is when spawning must occur.

Three generations of oocytes could be distinguished in the samples collected, as shown in Figure 5. Present are: (1) the oocytes which began growth in 1959–60 and would have been spawned in 1961, (2) those which began growth in 1960–61 and would have been spawned in 1962, and (3) part of those which would have begun growth in 1961–62 and would have been ready for the spawning period of 1963. In the November 11 sample from Cape Royds, all three of these oocyte generations could be distinguished; the oocytes of the 1961 spawning season had matured into ova ready for spawning, the oocytes for the 1962 spawning season had mostly reached one-third to one-half the diameter of full growth, and a few oocytes of the 1963 spawning season had just begun growth.

It is interesting to note that unlike the samples from Cape Evans and Cape Royds, there were many more small oocytes than large oocytes in the ovaries of the Turtle Rock sample (Fig. 4). The presence of a large number of small oocytes without a corresponding large number of the larger oocytes which had begun growth 8 to 12 months earlier suggests that many of the oocytes in specimens at Turtle Rock began growth only to be destroyed before they were a year old.

The individuals of the February 7 and April 5 samples were injected through the peristome with sea water saturated with KCl soon after collection. Two males and one female of the February 7 sample so treated spawned a few mature gametes but none of the individuals of the April 5 sample spawned. Some of the ova were fertilized when mixed with the spawned spermatozoa. The resulting zygotes were kept in sea water in a refrigerator at − 1.5 ± 1° C., but after a few unequal, abnormal-appearing cleavages, division stopped. Inasmuch as no mature ova were seen in the histological sections of the spawning female, it is uncertain whether the few ova obtained were residual ova or a precocious development for the forthcoming season.

Organic constitution of the tissues

With respect to lipid, carbohydrate, Lowry protein and total nitrogen no difference was detected between gonadal tissue prepared by freezing and drying and by fixing with trichloroacetic acid and desiccator-drying. Protein determined by the Kjeldahl method, however, was always higher in the frozen, dried tissues than in the trichloroacetic acid-treated, dried tissues (Table I).

The Lowry method gave higher, though more variable, protein values than the Kjeldahl method (Table I). Holland (1964) attributed the low Kjeldahl protein values for the coelomic fluid of *Strongylocentrotus purpuratus* as the result of incomplete precipitation of protein. The protein values obtained by the Lowry method are thus considered more realistic than those by the Kjeldahl method.

The lipid, carbohydrate, protein and total nitrogen levels of the gonadal samples of *S. neumayeri* are given in Table II. No seasonal change in the percentages of any of these constituents could be detected. The February to May Cape Evans samples had about the same levels of these constituents as the November and December Cape Evans and Cape Royds samples. However, as noted previously, there was a definite increase between early February and early May in gonadal size as expressed by the gonad indices (Fig. 1, Table II), so that on an absolute basis, the amounts of all the constituents measured increased during this period.

There was much less carbohydrate in the gonadal samples from Turtle Rock than in those from Cape Evans and Cape Royds. The lipid level in the testis samples from Turtle Rock was also unusually low. The difference in carbohydrate levels in the gonads was probably dependent on local factors, not on seasonal change, because it was also noticeable between the December 6 Turtle Rock sample and the December 7 Cape Evans and Cape Royds samples.

Sexual differences were found in the lipid and total nitrogen levels of the gonads. The ovaries usually contained much more lipid than the testes, this difference being especially marked in the sample from Turtle Rock. The testes always contained more total nitrogen than the ovaries. Because there was essentially no sex difference in the amounts of protein in the gonads, the higher total nitrogen content of testes was attributed to higher non-protein nitrogen content in the testes than in the ovaries.

The gonads of the specimen collected on December 7 from Cape Evans had the highest lipid levels and the lowest total nitrogen levels of any specimen sampled.

As the gonads consisted mainly of nutritive phagocytes with only a few gonial cells, their sex could not be determined. They had a histologic appearance similar to the inactive testicular lobes shown in Figure 3B and C. However, the high lipid and low total nitrogen content in these gonads was characteristic of the ovaries of the other samples.

Table III gives the lipid, carbohydrate, protein and total nitrogen levels in the samples of test, gut and gut contents. Although most of the specimens used for these determinations came from Turtle Rock, no differences could be detected between the Turtle Rock specimens and those from Cape Evans and Cape Royds. The body wall, with its high amount of calcium carbonate, contained very low percentages of organic constituents. The gut tissue was similar to the gonadal tissue of the Turtle Rock sample. The gut contents, consisting of partly digested pellets of seal feces and diatoms, contained lower percentages of lipid, carbohydrate and total nitrogen than the gut tissue.

TABLE III

*Organic constitution of the test, gut and gut contents of S. neumayeri collected between November and early December, 1961, from McMurdo Sound, Antarctica**

	Lipid	Carbohydrate	Lowry protein	Total nitrogen
Test	2.5 ± 0.4 (14)	0.6 ± 1.0 (14)	6.1 ± 2.4 (10)	1.2 ± 0.2 (14)
Gut	24.9 ± 5.3 (14)	3.0 ± 0.5 (12)	35.9 ± 6.8 (8)	8.7 ± 0.8 (14)
Gut contents	12.1 ± 7.0 (7)	2.0 ± 0.7 (8)	—	3.0 ± 2.3 (7)

* The tissues were fixed with trichloroacetic acid. Mean values are given in per cent dry tissue weight with one standard deviation. The number of animals sampled for each value is given in parentheses.

DISCUSSION

The common Antarctic echinoid *S. neumayeri* is similar to regular echinoids in other parts of the world in many respects besides appearance. Specimens often cover their tests with debris as do many other echinoids (Nichols, 1964). Like many other echinoids that have been studied (Hyman, 1955, pp. 558–589; Nichols, 1964), *S. neumayeri* is largely herbivorous, feeding mostly on diatoms. In this respect it resembles the Californian deep-sea echinoid *Allocentrotus fragilis* (Boolootian *et al.*, 1959; Giese, 1961). In both species this diet is probably the result of the ready availability of diatoms. The occasional seal-feces diet of *S. neumayeri*, however, is unique for an echinoid, but the omnivorous Antarctic asteroid *Odontaster validus* also feeds on seal feces when these are available (Pearse, 1965).

S. neumayeri appears to have relatively well defined reproductive periodicities as do many other echinoids (*e.g.*, as reported by Moore, 1934; Stephenson, 1934; Yoshida, 1952; Harvey, 1956; Fuji, 1960; McPherson, 1965; Holland and Giese, 1965). Even the tropical echinoid *Stomopneustes variolaris* shows periodic differences in breeding intensity though mature gametes occur all year (Giese *et al.*, 1964). The oogenic growth period of well over a year in *S. neumayeri* is similar to that of the Californian echinoid *Strongylocentrotus purpuratus* (Holland and

Giese, 1965). Many echinoids besides *S. neumayeri* also have large numbers of nutritive phagocytes in the gonads (*e.g.*, Caullery, 1925; Fuji, 1960; Holland and Giese, 1965) but in the gonads of *S. neumayeri* these cells seem to be especially abundant.

The differentiation and beginning of growth of the oocytes of *S. neumayeri* probably occur between November and May. This period is the austral summer when phytoproduction occurs (Littlepage, 1965). However, in December the sea temperature increases slightly in McMurdo Sound (Littlepage, 1965) and, as with the asteroid *O. validus* (Pearse, 1965), it is impossible to tell at present whether the increased temperature or phytoproduction, or neither or both, may play a role in synchronizing gametogenesis.

The gonads of *S. neumayeri* grow in size during the summer period of phytoproduction. Part of this growth is undoubtedly attributable to active gametogenesis, but part also appears to be the result of accumulation of nutrients in the nutritive phagocytes of the gonads. Because oocyte growth continues in the winter when no phytoproduction occurs, stores in the nutritive phagocytes must then be utilized for gametogenesis. The nutritive phagocytes thus appear to serve the same function as the pyloric caeca in the asteroid *O. validus* (Pearse, 1965).

S. neumayeri spawns sometime between May and December. This period is the austral winter and spring when there is little or no phytoproduction (Littlepage, 1965). The relatively small size (about 125 μ in diameter) of the ova of *S. neumayeri*, however, suggests that this species develops indirectly with feeding echinoplutei. Mortensen (1913), moreover, has described echinoplutei from Antarctica (Gauss Berg, 65° S., 90° E.) as those of *S. neumayeri*. These larvae were collected between December and early March, and those collected between February and March were in late stages of development. MacBride and Simpson (1908) also described two metamorphosing larvae (*Echinopluteus antarcticus*) collected from McMurdo Sound in January and February which Mortensen (1913) believed were those of *S. neumayeri*. *S. neumayeri* therefore probably has feeding echinopluteus larvae which occur in the summer even though spawning occurs in the winter or spring. As previously reported (Pearse, 1962a, 1962b, 1965), the asteroid *O. validus* spawns in mid-winter at McMurdo Sound, its embryos develop very slowly, and the feeding bipinnaria larvae are consequently present in the spring and summer months when plant food is available. It seems probable that this pattern of winter or spring spawning, very slow embryonic development, and the occurrence of feeding larvae in the summer when plant food is available characterizes *S. neumayeri* also.

Although *S. neumayeri* is very common in McMurdo Sound, and most likely has an echinopluteus larva, plankton samples taken bi-weekly or monthly from McMurdo Station during all of 1961 failed to reveal any echinoplutei (J. L. Littlepage, personal communication). If, indeed, *S. neumayeri* has an echinopluteus larva, these larvae, like those of *Odontaster validus* (Pearse, 1965), must be mostly demersal in habit. In this respect, it is noteworthy that 40 of the 46 specimens of *S. neumayeri* echinoplutei that Mortensen (1913) records were collected from depths of 100 m. or more.

The organic constitution of the tissue of *S. neumayeri* is not unusual in comparison with that of other echinoids studied (Giese, 1966). The levels of lipid, protein and total nitrogen in the gonads, gut and test tissues are very similar to those of other echinoids. Also, as in other echinoids, the ovaries of *S. neumayeri* contain more lipid than the testes, probably because it is accumulated in the oocytes. On the other hand, as in other echinoids, there is more non-protein nitrogen in the testes than in the ovaries, presumably because of the higher DNA level in the sperm. The carbohydrate level in the gonadal tissues of *S. neumayeri* from Cape Evans and Cape Royds is considerably higher than in most other echinoids, including *Strongylocentrotus purpuratus*, *S. franciscanus* and *Allocentrotus fragilis* (Giese, 1966), but it is somewhat lower than that reported in *Echinus esculentus* by Stott (1931).

Several differences were noted in this study between the specimens of *S. neumayeri* from Turtle Rock and those from Cape Evans and Cape Royds. The specimens at Turtle Rock were much smaller and their gonads contained much less carbohydrate than those from Cape Evans and Cape Royds. Moreover, there appeared to be considerable breakdown of oocytes in the ovaries of the animals from Turtle Rock while little or no oocyte breakdown occurred in the ovaries of animals from Cape Evans and Cape Royds. These differences among the populations of *S. neumayeri*, suggesting less favorable conditions at Turtle Rock, are in accord with other differences noted among the fauna of Cape Evans and areas further south in McMurdo Sound. Thus, the gastropod *Neobuccinum eatoni* was abundant at Cape Evans but was not seen at McMurdo Station which is south of both Cape Evans and Turtle Rock, and the nemertean *Lineus corrugatus* was smaller and contained less carbohydrate at McMurdo Station than at Cape Evans (Pearse and Giese, 1966). The asteroid *Odontaster validus* was smaller, less abundant and contained less red-orange integumentary pigment at both McMurdo Station and Turtle Rock than at Cape Evans and Cape Royds (Pearse, 1965). Further, the pyloric caeca of *O. validus* at Cape Evans increased in size by accumulating lipid, carbohydrate, protein and a green chlorophyll derivative during the summer months, while no such changes occurred in the pyloric caeca of *O. validus* at McMurdo Station. Many more gametes were also produced in the specimens of *O. validus* at Cape Evans than in those at McMurdo Station. From a consideration of the differing seasonal amounts of ice cover in McMurdo Sound, Pearse (1965) suggested that much more phytoproduction occurs at Cape Evans and areas further north than in more southerly areas such as at Turtle Rock and McMurdo Station. The differences noted among the populations of *S. neumayeri* in the present study, as well as those among the populations of *N. eatoni*, *L. corrugatus* and *O. validus*, suggest that the differences in phytoproduction are probably reflected in the fauna.

SUMMARY

1. The common Antarctic echinoid *Sterechinus neumayeri* (Meissner) feeds mostly on diatoms, although such things as seal feces are also used for food when available.

2. Specimens of *S. neumayeri* in McMurdo Sound have discrete reproductive periodicities as do most echinoids in other parts of the world. Oocyte growth begins in the summer between November and May, the oocytes take from 18 to 24 months to reach maturity after beginning growth, and spawning occurs sometime

in the winter or spring between May and December. The embryos presumably develop slowly into demersal echinopluteus larvae in the summer when plant food is available.

3. Nutrients seem to be accumulated in the nutritive phagocytes of the gonads during the summer when phytoproduction occurs, and the gonads increase in size during this period. These nutrients are probably utilized during the winter to maintain a relatively constant rate of gametogenesis.

4. The lipid, carbohydrate, protein and total nitrogen levels in the gonads, test, gut and gut contents of *S. neumayeri* are similar to those of other echinoids, although the carbohydrate content in the gonads is generally higher than in most other echinoid gonads that have been studied.

5. Differences among the populations of *S. neumayeri* in McMurdo Sound support an earlier suggestion that food conditions are better at Cape Evans and farther north than they are south of Cape Evans.

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GENE DEPENDENCY OF VEGETALIZATION IN SEA URCHIN EMBRYOS TREATED WITH LITHIUM

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The polypeptide antibiotic actinomycin complexes with DNA in such manner that the DNA-dependent RNA-synthesis is suppressed. The replication of DNA, on the other hand, is not, or only slightly affected by actinomycin D (see Reich and Goldberg, 1964). This antibiotic has therefore become a widely used tool in experiments aiming at the analysis of the importance of the transfer of genetical information in synthetic processes with cells or cell components. Different steps of synthetic processes form the basis of embryonic differentiation. Their "gene immediacy" (Davidson *et al.*, 1963) may be analyzed by interrupting the gene transcription with actinomycin.

Actinomycin was used, for example, in studies on the sea urchin development by Gross and Cousineau (1963), by Lallier (1963) and by the present writers (Markman, 1963; Markman and Runnström, 1963), in studies on amphibian development and regeneration in *Acetabularia* by Brachet *et al.* (1963). For more recent work reference may be made to Denis (1964) and Zetsche (1964).

In the studies of Markman and Runnström, isolated animal halves of embryos of *Paracentrotus lividus* were exposed to rather low concentrations of actinomycin D (5 μg./ml.). In the animal halves kept as control in normal sea water an animalization occurred as usual (see Hörstadius, 1935). This means an increased extension of the acron region, consisting of cylindrical cells carrying the stereocilia (about 70 μ long), forming the so-called ciliary tuft. Its extension gives a certain semi-quantitative measure of animalization. This depends on a rupture of the normal balance between the intercepting animal and vegetal gradients, the presence of which is supported by numerous experiments (Hörstadius, 1935, 1949; Runnström, 1933, 1954, 1964). Conversely, in the animal halves exposed to actinomycin, the animalization was much less pronounced, the acron and ciliary tuft hardly extending beyond the confines found in whole normal embryos. A certain although very weak tendency to formation of mesenchyme cells prevailed at the vegetal pole of the isolated animal half. The interpretation must be that transfer of genetical information is involved in the animalization of the animal half. Owing to the existence of the animal and vegetal gradients, a continuous system of different levels is formed. Gradually, an interaction between the cytoplasm and the nuclei intensifies which leads to a selective induction of gene activity. According to our view, the selection depends on the level at which the single nuclei are located in the double gradient system. The induction of gene activity leads to a certain unification of the levels to different discontinuous zones (Runnström and Immers, 1966). This process is blocked if the DNA-dependent formation of RNA is suppressed by actinomycin.

The weakening of the animal differentiation in the experiment referred to should thus depend on the block exerted by actinomycin against the production of messenger RNA, serving as template for proteins characteristic of the most animal zone of the embryo. With a different procedure Lallier (1963) demonstrated also the gene dependency of the animal differentiations.

In the present paper the question is raised as to whether transfer of genetical information is a prerequisite also for vegetalization. This has been brought about by the classical method of Herbst (1892) : exposure of the embryos to sea water to which LiCl has been added. It will be shown in the following that actinomycin counteracts the vegetalizing effect of lithium. There is an apparent divergence between this result and those briefly presented by Lallier (1963).

Material and Methods

The material used was *Paracentrotus* (*Pa.*) *lividus* from the Gulf of Naples. The methods of removing the gametes, washing the eggs, etc. were the same as previously used by these writers. The lithium was of the grade *pro analysi.* The actinomycin D was a gift from the research laboratories of Sharp and Dohme. In each experiment eggs from one female, fertilized with sperm from one male, were used. The volume of the test samples was 6 ml. with the same number of eggs in each of them (estimated to about 2000 eggs per ml.). After a certain time of exposure to lithium, actinomycin, or mixtures of both, as much as possible of the supernatant fluid was removed from the sedimented embryo by means of a pipette connected with a water pump; pure sea water was added to give a volume of 18 ml. After sedimentation of the eggs the same procedure was repeated. In this way the concentrations of the tested substances were reduced in the culture medium to less than 1% of the concentration prevailing during the time of exposure. The concentration after washing was more than 10 times below the limit at which any action could be traced.

Survey of the Main Results

Preliminary experiments were carried out with lithium concentrations varying between 0.033 and 0.049 *M* lithium; the concentrations of actinomycin D were 12.5 or 16.5 μg./ml. In the experiment pertaining to this report the exposure occurred during the cleavage and blastula stage (3–8 or 9 hours after fertilization). Variations in the degree of response depended as usual not only on the mode of exposure but also on the different susceptibility of materials from different batches of eggs.

It seemed suitable to use a lithium concentration which gives a clearly distinguishable but limited vegetalization. This should reduce the after-effect of pooled lithium on the embryos after return to normal medium. In the final procedure the eggs were kept for three hours after fertilization in pure sea water. The egg suspension was then diluted so as to obtain the following for test samples:

C_0	Egg suspension in sea water
C_{act}	Egg suspension in sea water with 12.5 μg./ml. actinomycin D
Li_0	Egg suspension in 0.033 *M* lithium
Li_{act}	Egg suspension in 0.033 *M* lithium with 12.5 μg./ml. actinomycin D

In Table I, one particular experiment with *Pa. lividus* (N 65, exp. 77) is recorded in a more detailed way. The exposure lasted for 5.5 hours (from 3 to 8.5 hours after fertilization). At different intervals embryos from the four test samples were fixed in neutral 4% formaldehyde and subjected to a detailed examination. A survey of certain features of the embryos is given in Table I under A-D. Reference

TABLE I, A–D

Survey of features of embryos of Pa. lividus treated according to scheme given in the text

A. Embryos fixed 22 hours after fertilization.	
C_O	Bilateral gastrulae (prisma stage).
C_{act}	Early stage of gastrulation. Primary and secondary mesenchyme cells in vicinity of the archenteron.
Li_O	Archenteron invaginated. Bilateral symmetry of the ectoderm is indicated. Primary mesenchyme cells arranged in a ring, the position of which was somewhat more animal than in C_O.
Li_{act}	No, or slight invagination of archenteron. Bilateral symmetry of the ectoderm only slightly indicated. Smaller primary (diameter *ca.* $5\ \mu$) and larger secondary mesenchyme cells arranged in two ventro-lateral groups.
B. Embryos fixed 27 hours after fertilization.	
C_O	Early plutei; pigment cells have appeared.
C_{act}	Gastrulae at the same level of differentiation as in *A* C_O, but bilateral symmetry more pronounced, and acron more extended than in *A* C_O. Acron participates in the formation of the ciliary band. Tendency to accumulation of primary in ventral, and of secondary mesenchyme cells in dorsal direction (*cf.* Markman, 1963, Fig. 1). No pigment cells.
Li_O	Vegetalization clearly indicated. The two attachment zones of primary mesenchyme were displaced in varying degree in the animal direction, their position often asymmetrical. Acron weakly differentiated. In 40–50% of the embryos the archenteron, in particular its animal region, was enlarged (Fig. 2). The archenteron could be completely invaginated but the invagination often was incomplete, forcing the anal ectoderm also to protrude (Fig. 3). Beside a main archenteron of normal size, accessory small archentera were sometimes present (Figs. 4 and 5).
Li_{act}	Gastrulae. In contradistinction to Li_O, archenteron of normal proportions. A tendency prevailed to assemble the primary mesenchyme cells in ventral and the secondary ones in dorsal direction (*cf.* C_{act}). No, or few pigment cells.
C. Embryos fixed 53 hours after fertilization.	
C_O	Plutei with four arms. Ciliary tuft replaced by motile cilia. Stomodeum united with oesophagus. Pigmentation normal. Beginning involution. Distance end of apex to top of anal arms: $230 \pm 13\ \mu$ ($n = 10$).
C_{act}	Embryos without arms, acron relatively larger than in C_O. Ciliary tuft of normal or slightly extended type still present. Relative to C_O, the intestine was under-dimensioned and incompletely subdivided into compartments (Figs. 6 and 7). Bilateral skeleton rudimentary. Number of pigment cells has increased. Apical region short. Animal-vegetal distance $164 \pm 2.6\ \mu$, ($n = 12$).
Li_O	Plutei; a high percentage of the specimens with a varying degree of slight vegetalization, as specified under *D*.
Li_{act}	Embryos with no, or rudimentary arms, no vegetalization. The outer profile different from C_{act}, in particular by the formation of a proportionate concave oral field (Fig. 8), sometimes with a stomodeum. The intestine subdivided into its three compartments. Ciliary tuft present in a number of the embryos (Fig. 8), but may be absent. Skeleton better developed than in C_{act}; oral and anal rods rudimentary.

Table I—(*Continued*)

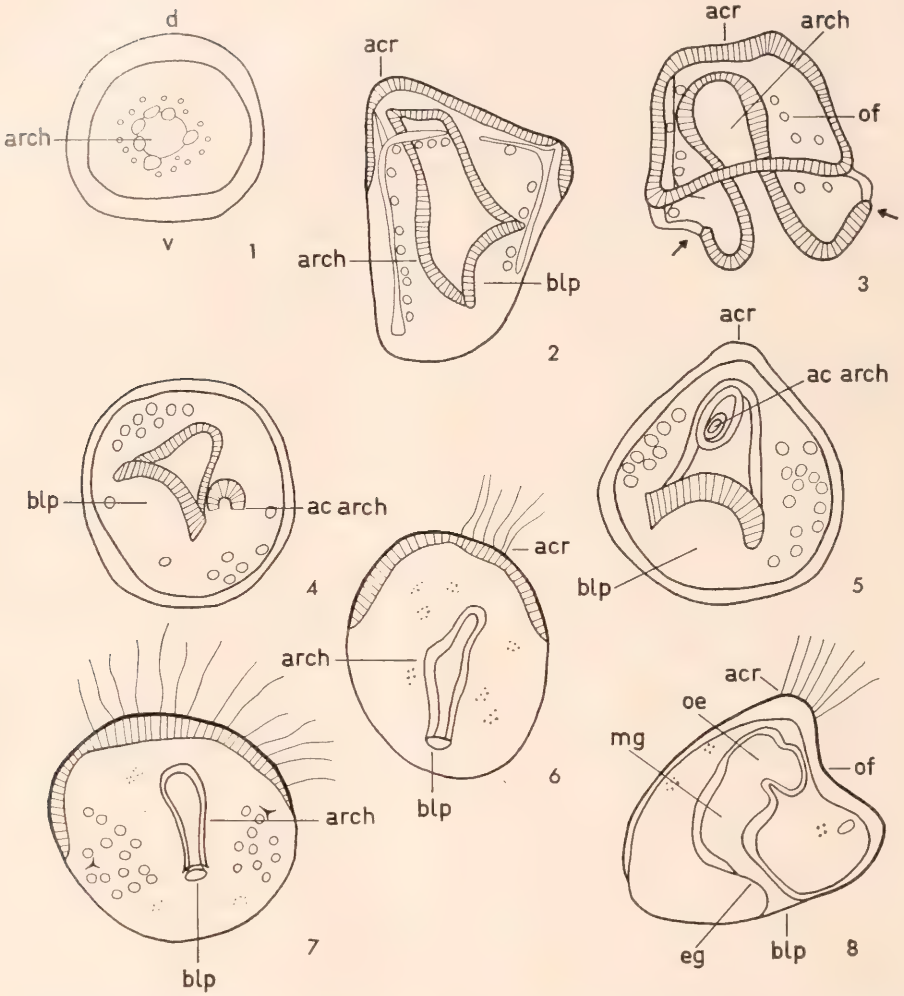
D.	Embryos fixed 70 and 79 hours after fertilization. No observable changes during this interval; material may thus be regarded as a uniform one.
C_0	Plutei in involution.
C_{act}	Rudimentary plutei in involution. No further differentiation of the intestine has occurred. Acron has often decreased after stage *C*, but ciliary tuft is often present.
Li_0	Plutei with elongated apical region; apical rods with club-like ends; these rods often crossed (Fig. 9) in a distal region. In contrast to the vegetal region of the oral field, its more animal region (the "oral lobe") often was reduced and asymmetrical. Of 166 closely examined larvae: (1), 36% were normal, or almost normal plutei; (2), 20% showed a reduced and (or) asymmetrical oral lobe, but archenteron of normal size; (3), 34% had to a varying extent reduced oral lobe and an enlarged and partially evaginated intestine (Figs. 9 and 10). In $<0.5\%$ typical lithium larvae with strongly reduced ectoderm and completely evaginated archenteron. Pigmentation.
Li_{act}	Of 235 embryos examined: (1), 88% were small plutei with rudimentary arms and blunt apical region (Fig. 11); (2), 12% were plutei with more developed arms (Fig. 12). Pigmentation.

is also made to Figures 1–12. Besides the observations on fixed material, living embryos were examined. Seventy-nine hours after fertilization the experiment was interrupted by fixation of the remaining material.

In a forthcoming paper Markman will report about quantitative autoradiographic studies of the incorporation of nucleic acid precursors in the presence of actinomycin. These measurements indicate that the exposure to actinomycin caused a strong inhibition of the RNA synthesis also in the present experiments. In experiments with *Psammechinus* (*Ps.*) *miliaris* from the vicinity of Kristineberg Zoological Station, Markman found results similar to those obtained in the present work on *Pa. lividus.*

Some Details and Comments Concerning the Morphogenesis Following Weak Lithium Treatment

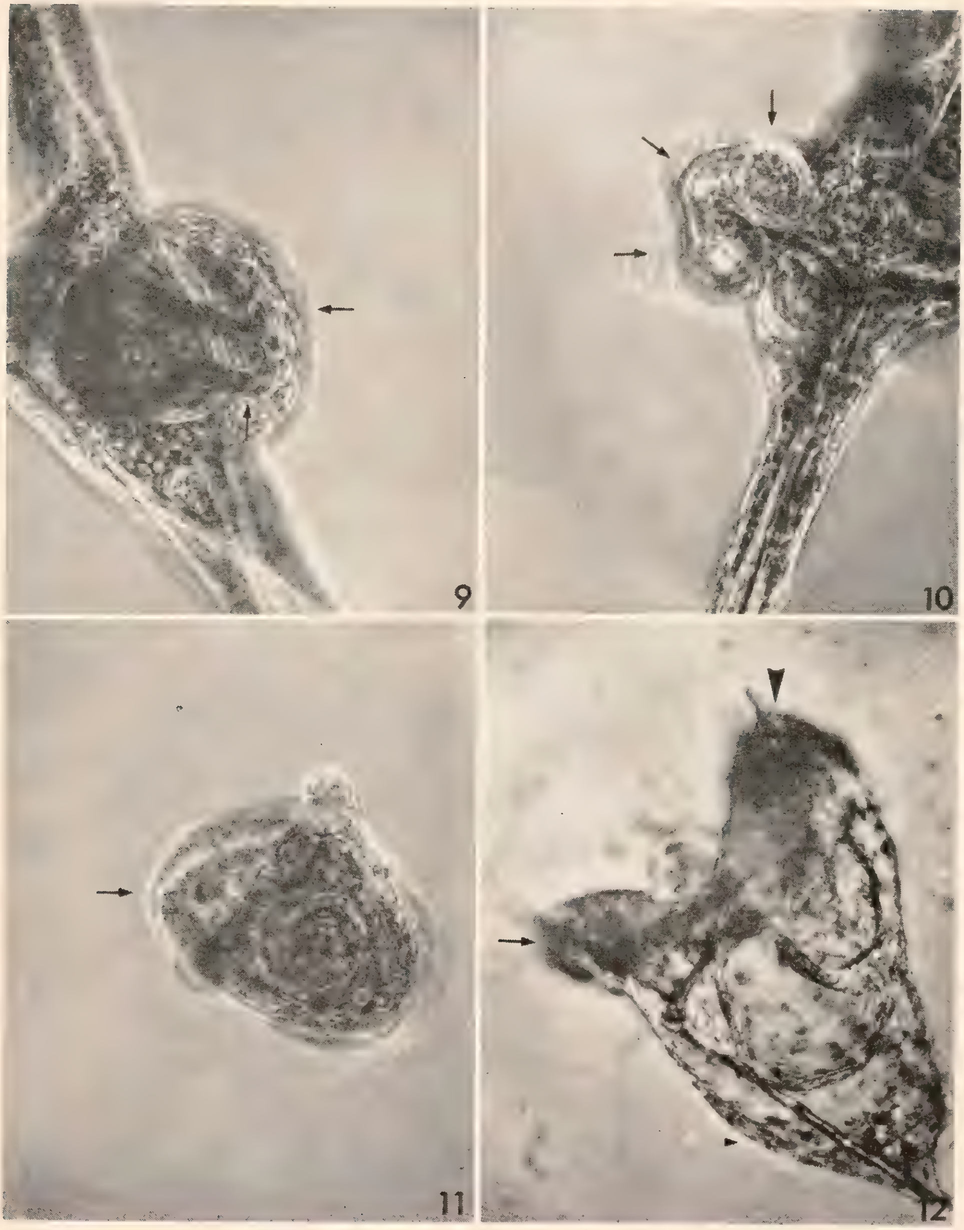
The embryos which have been exposed for 5.5 hours to sea water containing 0.033 *M* lithium showed a gamut from normal or almost normal plutei, plutei with defective animal oral regions to those with moderately enlarged archenterons. At the concentration of lithium and time of exposure used, only very few ($<0.5\%$) were typical lithium larvae with completely evaginated archenteron and strongly reduced ectoderm (Table I D, Li_0). The moderate enlargement of the archenteron was confined to its animal posterior region, as shown in Figures 2 and 3 (Table I B, Li_0). Of particular interest is the formation of smaller accessory invaginations (Figs. 4 and 5). In the present experiment the zone derived from the border regions between veg_1 and veg_2 (see Hörstadius, 1935) often seems to fluctuate with respect to its state of differentiation. Either the animal or the vegetal pathways dominate at about the same animal-vegetal level. Similar fluctuating states were observed by Lindahl (1936) in embryos from eggs which were pretreated with thiocyanate in calcium-free medium, fertilized in normal medium and thereafter transferred to sea water containing lithium. According to Hörstadius (1936), the fluctuations are also found when isolated animal halves are subjected to a somewhat delayed treatment with lithium.



Designation in FIGURES 1–8: *ac arch,* accessory archenteron; *acr,* acron; *arch,* archenteron; *blp,* blastopore; *eg,* endgut; *mg,* midgut; *oe,* oesophagus; *or,* oral field. Reference is made to Figures 1–8 in Table in the following places: *A,* Li_{act} (Fig. 1); *B,* Li_o (Figs. 2–5); *C,* C_{act} (Figs. 6–7); *C,* Li_{act} (Fig. 8) Ca. 250 ×.

The additional archenterons were found also in the pluteus stage. They were never subdivided in more than two compartments, but more than one additional archenteron was sometimes observed. In one embryo of 79 hours, one additional intestine had two compartments, but another was not subdivided. Moreover, the midgut of the main intestine showed two buds, one in ventral, another in dorsal direction, indicating a fluctuating state even at a more vegetal level.

Figure 3 represents an embryo 27 hours old (Table I B, Li_0), in which the archenteron showed an obvious enlargement. Its most animal part had not invaginated but formed a field of cylindrical cells (delimited in Figure 3 by arrows).



Designations in FIGURES 9–12: Figure 9, *horizontal arrow:* ectodermic bulge with squamous epithelium; *vertical arrow;* enlarged intestine opening through the bulge; 320 ×. Figure 10, *vertical and horizontal arrow:* bulge; oblique arrow, opening of the intestine. 160 ×. See for Figures 9 and 10, Table 1; *D,* Li_0. Figure 11, *horizontal arrow:* oral field with stomodeum. 160 ×. Figure 12, *vertical arrow head:* oral lobe with rudiment of oral arms with projecting skeleton rods; *horizontal arrow:* anal arm with skeleton rod; *lower arrow head* (*small*): apical region with rods; 320×. See for Figures 11 and 12, Table I, *D,* Li_{act}.

This field surrounded the entrance into the invaginated more vegetal part of the archenteron. The field showed a sharp delimitation against a lower epithelium belonging to the apical ectoderm, whereas the oral ectoderm formed a typical oral field, surrounded by a ciliary band; no stomodeum had appeared, and a skeleton had been formed only at one side of the embryo. In further development, embryos similar to that represented in Figure 3 gave rise to the type of plutei represented in Figures 9 and 10. A bulge was formed at the anal side of the embryo. The hind gut penetrated through this bulge and opened to the exterior. In the embryo of Figure 9, the opening was surrounded by a rim of entoderm corresponding to a non-invaginated part of the archenteron; the greatest part of the bulge consisted, however, of squamous epithelium of apical (dorsal) character. This ectodermic region was thus considerably enlarged at the cost of more animal ectoderm. In particular, the oral lobe was strongly reduced.

In the embryo represented in Figure 10 the lobe (arrows) had a more cylindrical character. It was perforated by the intestine which opened to the exterior by a funnel-shaped region. In a further example, two ectodermic bulges were found. One of these had squamous epithelium; the intestine had its opening in the wall of this bulge. The other one consisted of a rather high cylindrical epithelium; the communication between the interior of this bulge and the general blastocoel was rather narrow. The oral lobe was almost suppressed in this embryo, which exemplifies how presence of oral lobe and that of ectodermic bulge tends to exclude each other.

The cylindrical character of the epithelium seems to indicate that the bulge has a more animal character than that corresponding to its level in the animal-vegetal gradient system. The fluctuating state in the border zone may thus involve both ectoderm and entoderm. Its cause must be a weakening of the control, normally exerted by the animal polar region. In the present experiment, this control was impaired by the exposure to lithium in a concentration which did not bring about a full predominance of the vegetal pathways. Under these circumstances a labile state is produced so that the differentiation may easily be switched in one direction or the other. This indicates that rather sharp threshold effects prevail in the differentiation along the animal-vegetal gradient system.

The data which have been discussed in the previous paragraphs recall the "anarchy of gradients," described by Runnström (1933). Under the present conditions, addition of actinomycin D (Table I, Li_{act}) prevents this anarchy. In one case of about 300 a bulge appeared in the test Li_{act}. This bulge was of ectodermic character and exerted an evident action on the secondary mesenchyme cells (see Runnström and Immers, 1966).

CONSIDERATIONS OF THE GENE DEPENDENCY OF THE LITHIUM EFFECT

Table I, Li_0 and the preceding section made clear that lithium in the concentration and time of exposure used (0.033 *M* in sea water for 5.5 hours) was able to bring about a distinct vegetalization. If, in addition, the test medium contained 12.5 μg./ml. actinomycin D, plutei arose which usually deviated from the normal by being smaller or by having somewhat shorter skeleton rods and arms than normal plueti. This obvious approach to normal development demonstrates that gene activation and transfer of genetical information is a necessary link in the vegetalizing action of lithium. A weak effect of lithium reveals itself as a defective differentiation of the oral lobe (Runnström, 1928; Mastrangelo, 1965). This indicates that lithium affects primarily the animal anabolic pathways. Thus the synthesis of the specifically animal proteins or protein complexes may be impaired during the lithium-sensitive period, which, according to Lindahl (1940) and Bäckström and Gustafson (1953), is about 3–9 hours after fertilization.

Actinomycin D was found to remove the fluctuating state discussed in the previous section. Gene activity is thus involved in the switches occurring in the border zone between ectoderm and entoderm.

Runnström (1928) showed that lithium has a condensing action both on egg surface and interior of the eggs. Ranzi and collaborators (for comprehensive review, see Ranzi, 1962) showed *i.a.* that the proteins of embryos vegetalized by treatment with lithium were less easily attacked by trypsin than the proteins of control embryos.

Centrifugation experiments showed that lithium delayed the stratification of cytoplasmic particles relative to the stratification obtained in control eggs (Ranzi, 1962, p. 232; Lallier, 1955).

It seems possible that lithium has an effect on intra- and intermolecular bonding in certain cytoplasmic proteins which increases their tendency to aggregation. This view was substantiated in model experiments by Ranzi and collaborators (see Ranzi, 1962, *e.g.*, p. 237). The effect of lithium involves a competition with potassium (Runnström, 1928; Lindahl, 1936).

Molinaro and Hultin (1965) found a high potassium optimum for the incorporation of amino acids in cell-free systems from *Pa. lividus*. When lithium substitutes for potassium the amino acid incorporation is very low. Mastrangelo (1965) found, however, no effect on the amino acid incorporation in whole embryos subjected to vegetalizing concentrations of lithium.

In the living embryo lithium must, in the concentrations used, interfere with some weak link in the chains of physical and chemical events involved in the differentiation of the embryo. The constriction experiments of Hörstadius (1938) indicate the great importance of diffusion processes in this connection (for discussion, see Immers and Runnström, 1965). The primary lithium effect may particularly impede the diffusion of the animalizing agents which may undergo an aggregation or (and) become bonded with other components of the cells. The specific inducing effect of the animalizing agents on gene transcription is thus inhibited and, as a consequence, the animal anabolic pathways are suppressed to varying extent.

Conversely, the vegetalizing agents would be more independent of, or more protected against the cytoplasmic changes caused by lithium. An unbalance in the animal-vegetal gradient system would thus arise. This unbalance is enhanced by transient exposure to anaerobic conditions or to uncoupling agents (for survey, see Runnström, 1964).

Structural changes in the nuclei have also been described as an effect of exposure to lithium (Runnström, 1928; Hagström, 1963). It seems, however, less probable that lithium could exert its effect directly on the gene transcription with the remarkable degree of specificity observed.

Figure 13 refers again to the elaborate experiment of Table I. *An* and *Veg* indicate here the centers of production of the animalizing and vegetalizing agents, and the wedges their concentration gradients towards the opposite poles. These agents, or at least their precursors, are certainly present before the fertilization, as indicated also by the data presented by Giudice and Hörstadius (1965). A later replenishment mediated by genes is, however, probable. Figure 13 shows also how the agents may have a different influence on equal genoms located at two different levels in the double gradient system. The ratio of concentrations of animalizing and vegetalizing agents at the two levels is different and this is supposed to cause the activation or repression of different genes or operons (Jacob and Monod, 1963).

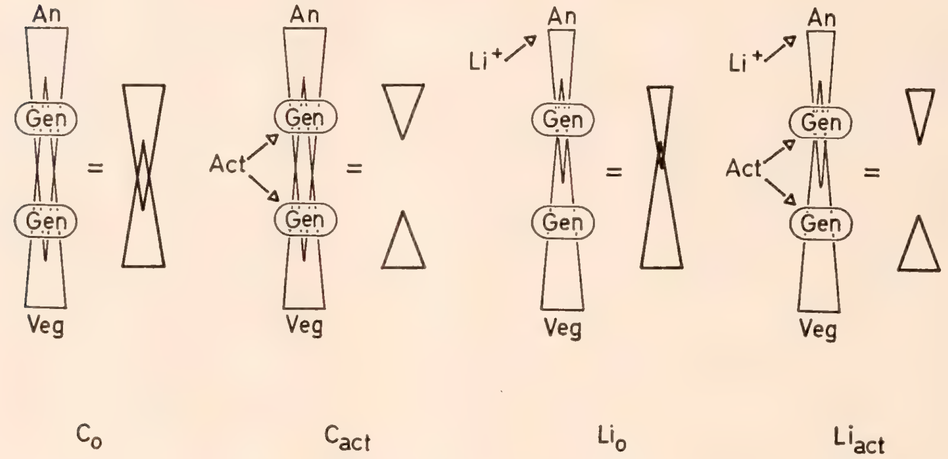


FIGURE 13. Diagrammatic representation of the state of chemo-differentiation in embryos exposed to sea water (C_o), to sea water with actinomysin D (C_{act}), to sea water with lithium (Li_o) and to sea water with lithium and actinomycin (Li_{act}). Each part of the diagram referring to one test (C_o, etc.) is divided into two parallel diagrams. The one to the left represents the gradients of animalizing and vegetalizing agents and their action on equal genoms at different levels during the exposure to the added substances. Actinomycin D blocks the transcription of information from the genom and removes thus, at least partially, the effect of the animalizing and vegetalizing agents. To the right, the extents of the animal and vegetal pathways are indicated in the embryos after transfer to normal medium. See, further, text.

To the right in each of the schemes C_0-Li_{act} in Figure 13, the results of the assumed interactions are symbolized. The wedges indicate here the relation between animal and vegetal anabolic pathways shortly after transfer to normal medium. C_0 shows the normal conditions in which the inducing animalizing and vegetalizing agents are in balance. In C_{act}, actinomycin D blocks the transfer of information both for the animal and the vegetal anabolic pathways. The consequence is a considerable inhibition of the differentiation of the whole embryo. When regulation processes begin after transfer to normal medium, the chances are about equal for the animal and the vegetal pathways, and an approach to a normal balance between these will occur.

In Li_0 the effect of the lithium treatment should be a decrease in the activity of the animalizing agents which induce the transfer of information for the animal anabolic pathways.

In Li_{act} as in C_{act}, actinomycin D blocks the transfer of information both for the animal and vegetal anabolic pathways. In this way, the inducing action of the vegetalizing agent is also excluded and the formation of additional entoderm which occurs in Li_0 is suppressed. This is the main piece of evidence to show that the vegetalization brought about by lithium is dependent on transfer of genetic information. One must presume that under the effect of lithium the action of vegetalizing agents is relatively increased even in the most animal region of the embryo, because the competition with animalizing agents is decreased. The vegetalizing agents reinforce then the vegetal anabolic pathways in the animal region. The presence of actinomycin D will, however, quench this effect of lithium and contribute to making the embryos subjected to the double treatment more balanced than those subjected to lithium alone.

In reality, C_{act} and Li_{act} are not as similar as should be expected from the outlined scheme. In the former, the archenteron was somewhat underdeveloped and poorly differentiated, even if a certain subdivision into compartments occurred. In still earlier stages the archenteron seemed condensed in an irregular way and had often no visible lumen. This may be due to the quenching effect of actinomycin D on the transfer of information necessary for certain secretions or components of the cell surface which are important for the capacity of the cells to slide against each other during mesenchyme formation and invagination of the archenteron. The impairment of the cell movements may have secondary effects on the functional, as well as on the morphological state of the archenteron. The moderating effects on acron and ciliary tuft may be reduced. The state of the mesenchyme and archenteron in C_{act} is to some extent reminiscent of that found in embryos which are raised in sulfate-free sea water (Runnström *et al.*, 1964; Immers and Runnström, 1965).

In Li_{act} the archenteron had more normal proportions than in C_{act}. The effect of lithium may not be completely eliminated following the combined treatment. A weak lithium effect may still be capable to counteract the tendency to animalization, prevailing in C_{act}. The consequence will be that the embryos, after transfer to normal medium, undergo a development in which the animal-vegetal balance is normal. The data of Giudice and Hörstadius (1965) exclude rather effectively that actinomycin D could combine directly with the vegetalizing agents and thus eliminate their action.

The discussion in this section may be reassumed by the following tentative conclusions. Lithium changes primarily the state of certain cytoplasmic proteins. The animalizing agents which induce transfer of information for the animal anabolic pathways are particularly sensitive to this primary lithium effect. This leads to a disturbance of the animal-vegetal balance which promotes the action of the vegetalizing agents. As a consequence the vegetal anabolic pathways are reinforced. This reinforcement is quenched by actinomycin D, which shows that gene activation and transfer of genetic information are involved in the vegetalizing action of lithium.

Lallier (1963) has presented data which seem to be at variance with those reported here. Thirty minutes after fertilization eggs of *Pa. lividus* were brought into sea water with 1.3×10^{-2} *M* lithium and 10 μg./ml. actinomycin D. The exposure lasted for 20 hours and thereafter the developing embryos were brought into normal sea water. Under these circumstances a stronger vegetalization was found after combined treatment than after treatment with lithium alone. Results of the kind obtained by Lallier may have the following explanation. Following return to normal sea water after a prolonged exposure to lithium in sea water, a certain pool of lithium may remain within the embryos which counteracts a regulation in direction of normal balance. If the embryos have been exposed to a prolonged treatment with both lithium and actinomycin D, also the latter may have an after-effect by its reaction with DNA. The transfer of information will at least be delayed, which tends also to hold back the regulation. The discrepancy between the above results and those of Lallier (1963) may not be regarded as removed by these suggestions. These writers have, however, considered that their experimental device would be most suitable to resolve the problem raised in the introduction. Furthermore detailed studies on the morphogenesis of the treated embryos, as well as measurements of incorporation of RNA-precursors, will be necessary in order to get more insight into the state of the embryos subjected to prolonged exposures.

The experimental part of this work was carried out at the Stazione Zoologica, Naples, during the spring of 1965. It is a pleasure to express our gratitude to the Director, Dr. Peter Dohrn, and to Dr. Luisa Tosi for their help and support. We acknowledge gratefully the financial help received from the Swedish Natural Sciences Research Council and the Swedish Cancer Society.

SUMMARY

1. Embryos of *Paracentrotus lividus* were raised in sea water (C_0); in sea water with 12 μg./ml. actinomycin D (C_{act}); in sea water with 0.033 M lithium (Li_0) and in sea water with both 0.033 M lithium and 12 μg./ml. actinomycin D (Li_{act}). The embryos were transferred into the media at the 8-cell stage and exposed to these for 5.5 hours; thereafter they were returned to normal sea water. The embryos or larvae were fixed after different intervals of time as detailed in Table I. Living specimens were also observed. The lithium concentration and time of exposure used gave a weak but definite vegetalization in a high percentage of larvae (Table I, D, Li_0). The effect of lithium either involved merely a reduction of the animal-oral region (oral lobe) or was manifested also as an enlargement of the archenteron, in particular of its more animal (posterior) region. Accessory archentera or ectodermic bulges were often found in the border region between ecto- and entoderm. The differentiation seems here to be in a "fluctuating" state.

2. Previous results of Runnström, Ranzi and Lallier indicate that the primary effect of lithium increases inter- and intramolecular bondings in certain cytoplasmic components. The animalizing agents are assumed to be particularly sensitive to these changes which may block their diffusion. In this way, the normal animal-vegetal balance is disturbed in favor of the vegetalizing agents which are less affected by the structural changes.

3. The vegetalizing agents may directly or indirectly induce the activation of genes concerned with the vegetal anabolic pathways. When the action of these agents is enhanced in the state of unbalance caused by lithium, the predominance

of the vegetal anabolic pathways extends in animal direction. If, however, the transcriptions of genes are blocked by actinomycin D this extension of the region of vegetal pathways is inhibited (for a diagrammatic representation of lithium action on one side and the combined action of lithium and actinomycin D on the other, see Figure 13, Li_0 and Li_{act}). The blocking effect of actinomycin D does not depend on a direct reaction with the vegetalizing agents, as follows from data presented by Giudice and Hörstadius (1965). The inference is that gene activation is a link in the vegetalization following exposure to lithium.

4. Exposure to actinomycin D alone (Fig. 13, C_{act}) causes a tendency to animalization which is a secondary effect of retarded gastrulation. The development of the embryos becomes more balanced after exposure to the combined action than after that to actinomycin alone. A weak lithium effect balances the slight tendency to animalization which follows upon treatment with actinomycin D. Even after combined treatment, however, a certain inhibition prevails, manifested particularly in subnormal outgrowth of skeleton rods and arms.

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HYBRIDIZATION EXPERIMENTS WITH AN ALL–FEMALE FISH OF THE GENUS POECILIOPSIS[1]

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The sex-determining mechanism of the monosexual strains of *Poeciliopsis* is unique to vertebrate animals. Two such all-female populations have thus far been found among the 16 species that form this principally Mexican genus of poeciliid fishes (Miller and Schultz, 1959). Each of these all-female strains, *P.* Cx and *P.* Fx, is superimposed on a bisexual or normal species (*P.* C, now described as *P. lucida* by Miller, 1960, and *P.* F, still undescribed) and is dependent on the male of that species for fertilization.

Laboratory studies (Schultz, 1961) have provided the following hypothesis regarding the origin and perpetuation of monosexuality in *Poeciliopsis:*

1. Hybrid crosses between bisexual species indicate that the sex-determining mechanism varies in strength between different members of the genus; thus when a male from a species with a "strong" mechanism is mated to a female of a species with a "weak" mechanism, the progeny are predominantly male. From the reciprocal cross, however, predominantly females are produced.
2. It is believed that all-female "species" of *Poeciliopsis* originated in nature when a very "strong" female crossed with a "weak" male and so produced all female hybrid offspring. These now maintain themselves by continuously backcrossing with the weak male.
3. In a series of backcrosses one might expect such a mechanism to break down rapidly and to re-establish normal sex ratios—provided random segregation of chromosomes takes place. By using a marker gene, it was established that at least one chromosome is not transmitted through the egg; and it is possible that a large number or even all of the paternal chromosomes of each generation are lost during oogenesis. If the latter obtains, then, the ova receive only the maternal complement of chromosomes generation after generation, even though the all-female progeny do reflect characteristics of the father of each mating.

Exclusion of paternal traits from the eggs of *Poeciliopsis* Cx seems to be the female version of a similar mechanism for chromosome elimination in *Sciara* (fungus gnats), wherein all paternal chromosomes are pinched off in a tiny bud during spermatogenesis (Swanson, 1957).

The mode of inheritance of the monosexual *Poeciliopsis* should not be confused with that of *Poecilia* (*Mollienesia*) *formosa*, in which all-femaleness results from gynogenesis. In *P. formosa* the sperm of other species is essential to activate

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[1] This investigation was supported by National Science Foundation grants GB-2390 and G-24129.

development of the ova but there is no gametic fusion; therefore, the progeny are identical to the mother (Hubbs and Hubbs, 1932, 1946; Kallman, 1962).

If monosexuality in *Poeciliopsis* has evolved in the manner postulated, one might expect to find among the 16 bisexual species some that have sex-determining mechanisms sufficiently strong to counter-balance the all-femaleness. In previous studies it was demonstrated that *P.* Fx would hybridize with the allopatric *P. lucida* and that *P.* Cx would hybridize with the *P.* F.; but from both matings, exclusively female progeny were obtained. *P. lucida* and *P.* F are closely related; and, although mating tests demonstrate that there is a difference in the sex-determining potential of the two species, neither apparently has sufficient strength to topple the all-female mechanism.

Living in the Rio Mocorito with *lucida* and Cx is *P. latidens* Garman, a species that differs considerably from the mates of the all-female forms. This species when mated to Cx produces progeny of both sexes. All of the male progeny thus far have been sterile but the females are fertile.

In the 1961 study, only a single marker gene was available to trace the route of paternal chromosomes in matings with monosexual forms; and although inferences could be made, based on the consistence of maternal inheritance, it could not be demonstrated with certainty that any more than one paternally derived chromosome was consistently lost at the time the F_1 ova were produced. The great number of characters by which *latidens* differs from Cx and *lucida* provides the necessary tools for determining to what extent paternal chromosomes are transmitted through the ova in various hybrid matings.

MATERIALS AND METHODS

All of the specimens used in this study were descendants of fishes collected from the Rio Mocorito in the state of Sinoloa, Mexico, near the villages of San Benito and La Huerte by Miller and Schultz, 1959; Miller and Huddle, 1961; and Miller and Schultz, 1963. Gravid females were transported to the laboratory by air freight and upon arrival were isolated in two- and four-gallon aquaria. The young born to these wild females provided the progeny used in the mating experiments. Offspring produced from the various matings were all reared to approximately the same size (33 to 35 mm.) to facilitate quantitative comparisons. These were killed and fixed in 7% formalin and stored in 70% alcohol. The lower jaws were later removed, cleared in potassium hydroxide and stained in alizarin for osteological studies. Tooth measurements were made with an eyepiece micrometer. All quantitative comparisons were based on the t-test using 5% as the level of significance.

RESULTS

Sex ratios

Over the 5 years that Rio Mocorito fishes were maintained in the laboratory, 10 generations of *Poeciliopsis* Cx were produced from matings with *P. lucida* males. Of the 306 Cx young reared to maturity, not one was a male (Table I). There was no evidence of an unbalanced sex ratio in either of the two bisexual species involved in the study: (1) 44 *lucida* pairs in 13 generations produced 366 females and 376 males (1:1 ratio $\chi^2 = 0.136$) and (2) 11 *latidens* pairs in 5 generations produced 60 females and 52 males (1:1 ratio, $\chi^2 = 0.067$).

In the hybrid cross Cx × *latidens,* 6 pairs produced 87 F_1 progeny. Here, for the first time in the laboratory males were born to Cx mothers. The sex ratio, however, was far from 1:1 since 81 of these were females and only 6 were males. All males were produced from two females (5 from one and one from another) in spite of the fact that the non-male producers had substantial numbers of offspring.

TABLE I

Sex ratios of stock cultures and hybrid crosses

	Number of pairs involved	Offspring produced			Number of generations represented
		Females	Males	Total	
lucida × lucida	44	366	376	742	13
latidens × latidens	11	60	52	112	5
Cx × lucida	26	306	0	306	10
Cx × latidens	6	81	6	87	—
(Cx × latidens) × latidens	3	18	0	18	—
(Cx × latidens) × lucida	2	8	0	8	—

Backcrosses of the F_1 female to the *latidens* male were successful three times for a total of 18 progeny, all of which were females. Two matings back to the *lucida* male resulted in 8 offspring which were again all females.

Genitalia

Classification of poeciliid fishes depends largely upon the morphology of the male's copulatory organ, known as a gonopodium. This structure derives from a modification of the anal fin. Elongation and specialization of the fin rays of juvenile males in *Poeciliopsis* leads to the formation of an imperfectly closed tube—imperfect in that a narrow groove remains open along the left side. At the tip beyond the termination of the fin rays, there is a fleshy protrusion of the cutis. This structure varies in shape from one species to the next and probably serves as part of the holdfast mechanism during copulation. Rosen and Bailey (1963) in revising the family Poeciliidae emphasized the importance of the gonopodium and its internal skeletal supports, the suspensorium, in the systematics of the group. Although a comparison of suspensoria within the genus *Poeciliopsis* is yet to be worked out, there is little question that the tip of the gonopodium provides a useful taxonomic tool (Hubbs and Miller, 1954; Miller, 1960).

The genitalia of females, in contrast to males, has played an insignificant role in the classification of poeciliid fishes in general. In *Poeciliopsis,* however, the shape of the genital papillus and the distribution of melanin in this region are of considerable value for the separation of species. Extending from the base of the anus to the origin of the anal fin is a scaleless depression of soft tissue, the genital pit. The fleshy papillus with the genital opening at its tip arises from this pit as a more or less cone-shaped structure. All forms of *Poeciliopsis* dealt with in this

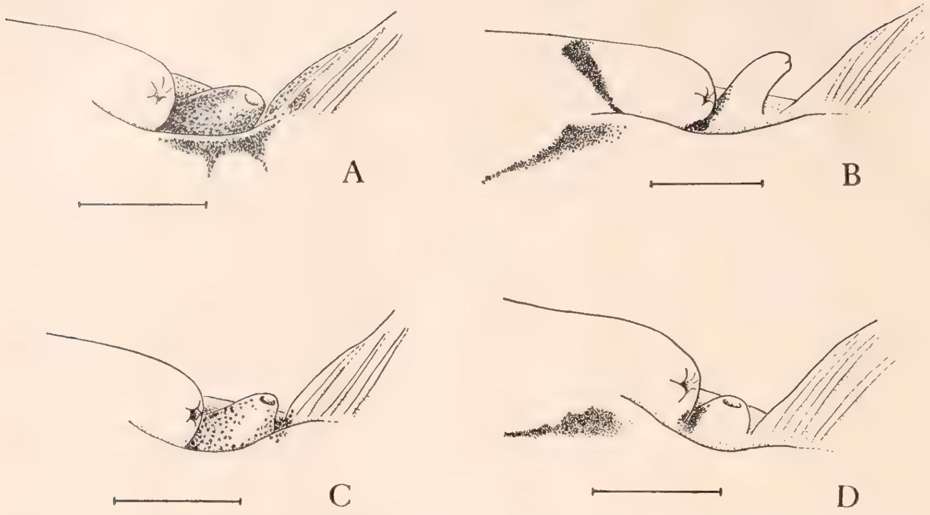

Figure 1. Genital region of *Poeciliopsis* females: A, *P. latidens;* B, *P. lucida;* C, Cx × *latidens* or Cx × *latidens* × *latidens;* and D, Cx or Cx × *latidens* × *lucida.*

study can be easily identified on the basis of the female's genitalia, a factor of no small importance in a group containing all-female fishes.

P. latidens. The genital pit and papillus of this species are so densely covered with melanin that the individual melanophores can be seen only in the peripheral region. This blackened area in *latidens* is more extensive than in *P. lucida,* Cx or the Cx × *latidens* hybrids (Fig. 1). It forms a large blotch that overflows the pit and extends laterally across the first row of scales and posteriorly on to the anal fin as far as the second or third fin ray and distally on the anal fin one-half to three-fourths the length of the first ray. Extending forward along both sides of the anal elevation are two horns of pigment that lie deep in the tissues. These are not well developed in live *latidens* females; in preserved specimens, they are usually not seen at all because of the opacity of the over-lying tissues. The papillus in live females resembles a squat volcano that practically fills the genital pit. Upon preservation, however, it becomes more slender, often flexed with the genital opening drawn nearly closed.

The structure of the gonopodium of *latidens* has been depicted in detail by Hubbs and Miller (1954). It is sufficient here to consider only the tip of the organ which hooks strongly downward, so that, viewed laterally it resembles the letter "J," fattened at the bottom by an unsegmented fleshy portion and supported on the inner curvature by the tapered fin rays (Fig. 2).

P. lucida. The genital papillus of *P. lucida* is a long, slender, finger-like process (Fig. 1). Development of melanin is minimal and restricted primarily to the pit region between the papillus and the anus; only a few melanophores extend on to the papillus where they are confined to the anterior surface. Scattered melanophores are occasionally found in the posterior part of the pit but by and large this area is silvery-white as is most of the papillus. Two horns of black pigment arise from the anterior margin of the pit and course upward and forward through the tissue on both sides of the anus, forming a V-shaped pattern near the surface. Anterior to the base of the anus a curved black line crosses from one arm of the V to the other. Although all of the other characters are present in preserved specimens, the pre-anal cross-bar is quickly lost due to the opacity of the overlying tissues.

The terminal rays of the *lucida* gonopodium are not curved as in *latidens*. There is, however, a fleshy unsegmented tip distinctive from that of *latidens* by an anterior projection described by Miller (1960) as a "retrorse hook" (Fig. 2).

P. "Cx." In this all-female form, distribution of melanin about the genital region is the same as in *lucida*, being most pronounced in the depression between the anus and the papillus, extending somewhat up the anterior side of the papillus but almost totally absent from the posterior part of the pit (Fig. 1). The all-female genitalia differs from that of *lucida* on two counts: (1) The papillus is short and fat instead of long and finger-like, and (2) the curved pre-anal cross-bar is lacking, though a pre-genital V does extend upward and forward along the sides of the anus.

Cx × *latidens* and Cx × *latidens* × *latidens*. No difference could be found between the genitalia of the F_1 and the backcross hybrids; hence, they are described as

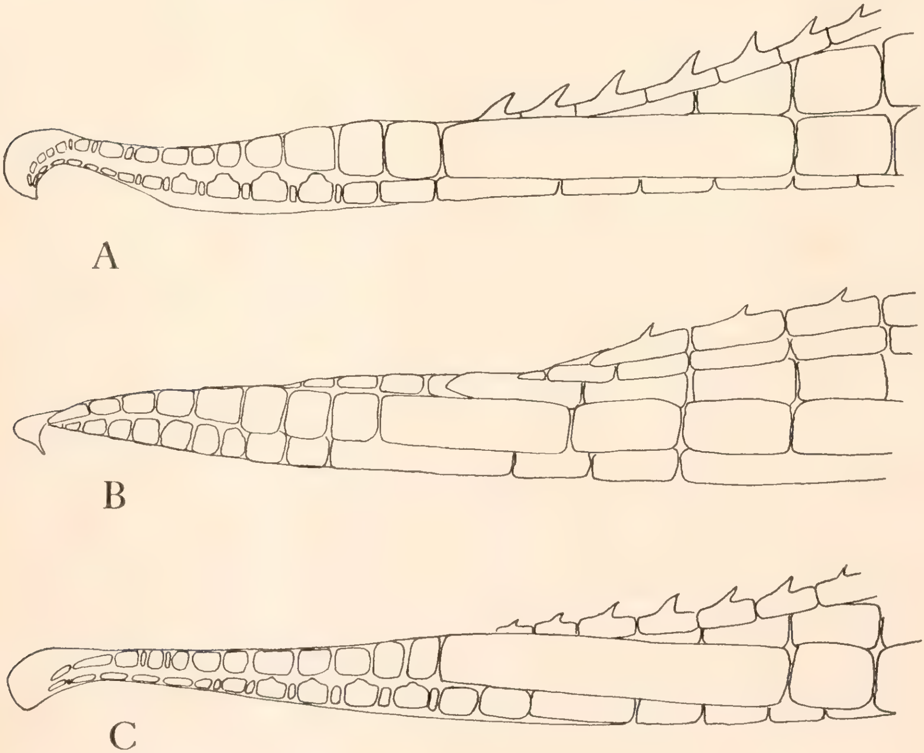

FIGURE 2. Gonopodial tips of (A) *Poeciliopsis latidens,* (B) *P. lucida,* and (C) Cx × *latidens* F_1 hybrid.

one (Fig. 1). The distribution of melanin varies among individuals but there is no problem distinguishing the hybrids from either Cx or *latidens.* At maximum development melanin is found scattered over most of the papillus and throughout the pit with some pigment sprinkled over the edge of the pit. A few melanophores dot the base of the anal fin near the pit but do not extend down the fin as they do in *latidens.* In the most densely pigmented individuals, the blackened area is not only less extensive than in *latidens* but the melanophores are more distinct and do not present a uniformly black coating. At minimal development the papillus is without melanin but unlike *Cx* or *lucida* there is always a sprinkling of melanophores in the posterior part of the pit. The shape of the papillus is not consistently different from that of *latidens.*

The tips of the gonopodia of the few hybrid males produced are less curved than those of *latidens* and lack the retrorse hook of *lucida.* The terminal rays curve only slightly and the fleshy tip viewed from the side curves more gently than in either *lucida* or *latidens.*

Mouthparts—lips, jaws, and teeth

The jaw bones, teeth and lips of *Poeciliopsis* prove to be among the most diagnostic characters available for separating members of the genus. As a matter of convenience, investigation was restricted to the lower jaw which can be easily removed, cleared, stained and examined under the microscope without damaging the rest of the specimen.

The teeth are arranged in two groups: (1) the large, outer teeth, and (2) the tiny, inner teeth. The outer teeth are planted firmly in the middle of the anterior face of the dentary bone. They curve upward over the face of this bone and turn sharply inward as they emerge from the flesh into the mouth. The shank of the outer tooth is rounded in cross-section and completely embedded in the tissues of the mouth. Most of the flattened portion or blade is also embedded with only the tip exposed. The shape and width of the blade vary from one species to the next. Behind the single row of outer teeth are one to many irregular rows of the tiny, inner teeth embedded on the dorsal surface of the dentaries where they run along the anterior margin and extend a short way down the outer margin of each jaw.

When viewed from above the cleared and stained dentary bone (Fig. 3) resolves itself into three prominences: (1) A large flat shelf upon which the inner teeth are secured. This thins out posteriorly to form an over-hanging margin with a contour that is species-specific. (2) There is a posterior wing forming the sides of the jaw; it has one groove on the outside for muscle attachment and one on the inside that receives the articular bone, and (3) an elbow projects from beneath the middle of the shelf and extends posteriorly and downward a short distance before making a right-angle mesial turn, terminating short of the midline.

The lips of some species are thick and rounded in cross-section but in others they are thin and flap-like. From a dorsal view they may be strongly curved, providing a streamlined snout, or they may run straight across, giving a squared-off appearance. The lower lip precedes the upper as is characteristic of other surface feeders.

P. lucida. The dentary bone of *P. lucida* forms a right angle at the anterior-

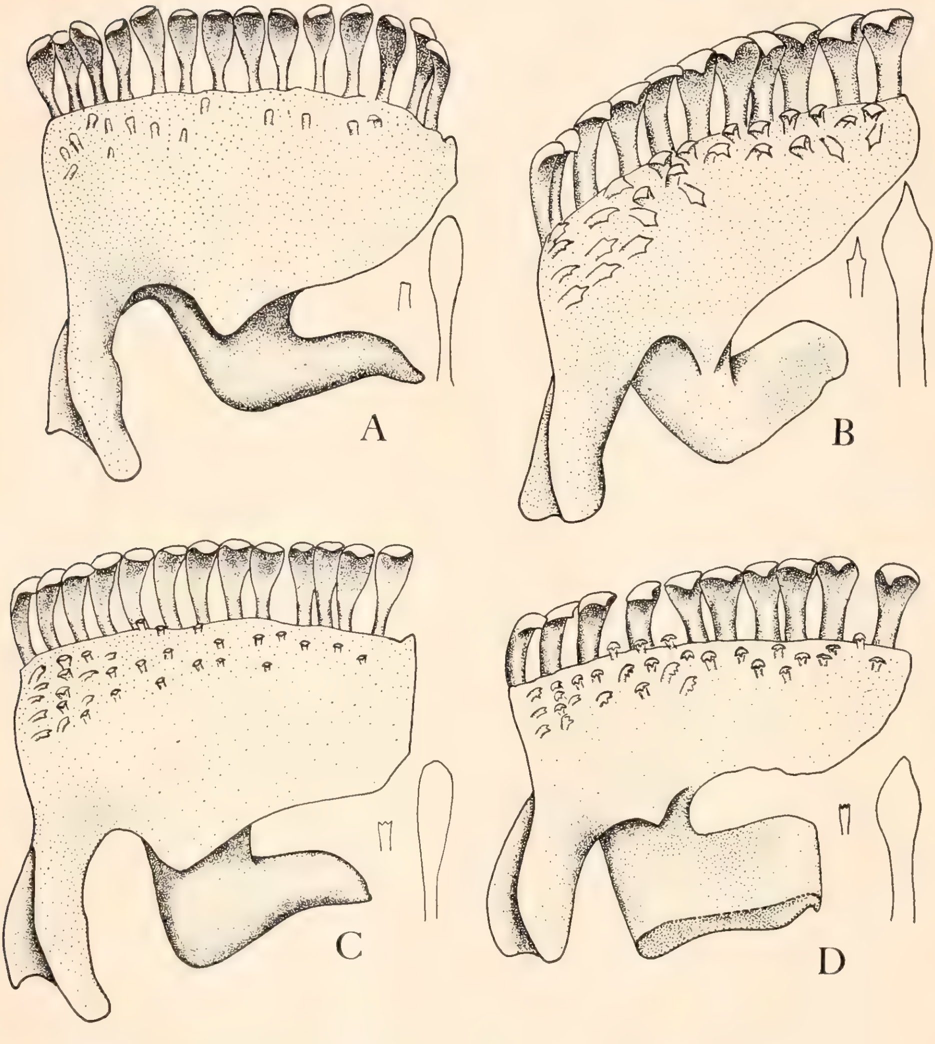

FIGURE 3. Left dentary bone of (A) *Poeciliopsis lucida,* (B) *P. latidens,* (C) Cx, and (D) Cx × *latidens* F_1 hybrid.

lateral corner; but as the anterior margin runs toward the midline it curves inward producing an indentation at the symphysis of the two dentary bones. The shelf that supports the inner teeth and forms the floor of the lower jaw is well developed and extends backward in a broadly curving, posterior-mesial over-hanging margin. The elbow, after making its right-angle turn, is rounded in cross-section and tapers toward the tip. The thin, straight lip-line is interrupted only by a deep median indentation.

The outer teeth are smaller (53.4 microns wide, Table II) and more numerous (average per lower jaw 29.07, Table III) than in any of the other forms of

TABLE II

Width in microns of outer and inner teeth. Horizontal lines separate groups significantly different ($p < 0.05$) from each other

Mating	Average maximum width of inner teeth	Average maximum width of outer teeth	Average minimum width of outer teeth
lucida × lucida	19.4 ± 0.46	53.4 ± 0.92	18.6 ± 1.8
Cx × lucida	24.2 ± 1.6	58.4 ± 0.6	21.6 ± 0.2
Cx × latidens × lucida	23.8 ± 0.8	58.0 ± 0.6	21.8 ± 0.2
Cx × latidens	33.2 ± 3.2	80.8 ± 4.2	40.8 ± 0.6
Cx × latidens × latidens	33.4 ± 0.5	79.4 ± 4.6	41.8 ± 0.8
latidens × latidens	37.2 ± 1.2	91.0 ± 1.4	44.8 ± 0.6

Poeciliopsis considered here. The tips of these teeth are mostly rounded or spatula-shaped; some form a slanted chisel but none are pointed or spear-shaped. The flattened portion or blade is extensive and about two and one-half times as wide as the short rounded shank. Not only are the inner teeth the smallest (19.4 microns) but they are also the least numerous of any of the forms studied (35.36). They are in the main poorly developed, being mostly peg-shaped and spatula-shaped with fewer than one-fourth of them suggesting the tricuspid condition.

P. latidens. The snout of this species is strongly curved. It has thick, full lips with no suggestion of a median indentation. Curvature of the snout stems not only from the fleshy elements but also from the skeletal structure. From the point of symphysis of the dentary bones the anterior margin sweeps back in a curve forming a 120° angle with the lateral margins—this in contrast to *lucida* and Cx, wherein the angle is 90°. The dorsal plate of the dentary does not have a well developed over-hang; rather, the posterior margin cuts diagonally up and across from the base of the elbow, eliminating the medial margin except for a narrow symphysis. In cross-section the terminal arm of the elbow is square.

The outer teeth are fewer (24.15) than in Cx or *lucida* but they are larger (91.0 microns). There is a heavy, long shank embedded in the flesh with a short spear-shaped blade. The inner teeth are large (37.2 microns) and well developed. These are tricuspid with the middle tine longer than the two outer tines. Often

TABLE III

Mean number of inner and outer teeth in lower jaw. Horizontal lines separate groups significantly different ($p < 0.05$) from each other

Mating	N	Outer	Inner
lucida × lucida	14	29.07 ± 0.71	35.36 ± 1.16
Cx × lucida	14	27.00 ± 0.47	66.13 ± 3.59
Cx × latidens × lucida	7	27.29 ± 0.35	61.14 ± 3.35
Cx × latidens	14	21.47 ± 0.46	64.33 ± 3.80
Cx × latidens × latidens	7	20.88 ± 0.56	76.14 ± 2.86
latidens × latidens	14	24.15 ± 0.67	60.54 ± 4.81

the outer tines are worn down to little more than a shoulder. There are nearly twice as many inner teeth (66.13) as in *lucida* but the average number is not significantly different from the rest of the forms considered here.

P. Cx. The shape of the lips and dentary bones of Cx resembles those of *lucida* in that their anterior-lateral margins form a square-cornered mouth. Except for a median indentation, somewhat less pronounced than in *lucida,* the lips and anterior margin of the dentaries run straight across. Posteriorly the dentary plate is even better developed than in *lucida.* The posterior over-hang rather than being rounded is squared off; thus the dentary plate is nearly rectangular. The elbow is rounded and tapers toward the tip.

The outer teeth do not differ greatly in shape from those of *lucida.* They are spatula to chisel-shaped, slightly wider (58.4 microns) and on the average slightly fewer (27.0). A short, slender peduncle-like shank and long blade are characteristic of both forms.

It is in the inner teeth that the primary differences are to be noted. In contrast to *lucida,* wherein most of the inner teeth are unicuspid, those of Cx are basically tricuspid. Fewer than one-fourth are of the *lucida* type, even though the specimens examined were sired by *lucida* males. The tricuspid condition here differs from that characteristic of *latidens* in that the middle tine is not greatly elongated; furthermore, these teeth are only about two-thirds the size of the *latidens* teeth (24.2 *vs.* 37.2 microns). The number of inner teeth in the lower jaw of Cx is nearly twice that of *lucida* (66.13 *vs.* 35.36) but not significantly different from *latidens* (60.54). These are arranged in two or three uneven rows along the anterior margin of the dentaries with some clustering along the lateral margins.

P. Cx × *P. latidens.* The snout of the Cx × *latidens* hybrid is rounded but less so than that of *latidens.* The moderately thickened lips show either no evidence of a median indentation or in some only a trace. Each of the dentary bones is slightly rounded both laterally and medially; there is, thus, a slight indentation at the symphysis, even though there is little reflection of this trait in the thick lips. The angle formed by the anterior-lateral margins of the dentaries is less pronounced (100°) than that of *P. latidens* (120°). In the hybrid, the shelf is narrow as in *latidens* but the posterior over-hang is better developed and has a rounded corner at the medial margin rather than a diagonal cut straight to the symphysis. The elbow, unlike that of *lucida,* Cx, or *latidens,* is broad and flattened.

The outer teeth of the hybrid resemble those of *latidens:* they have a thickset shank that comprises more of the tooth length than does the blade and there is a spear-shaped tip which is, however, less pointed than in *latidens.* The number of outer teeth in the lower jaw (21.47) is less than that of either parent (Cx—27.00 and *latidens*—24.15); whereas the width (80.8 microns), although intermediate (58.4 and 91.0 microns), lies closer to the dimensions of the wider *latidens* teeth.

The inner teeth are all tricuspid; and although their size (33.2 microns) more closely approximates that of *latidens* (37.2 microns), they lack the long middle tine and, hence, are shaped more like the smaller (24.2 microns) Cx type. Their average number per jaw (64.33) does not differ significantly from that of Cx or *latidens.*

Cx × *latidens* × *latidens.* The shape of the lips, dentary bones and teeth of the backcross progeny is identical to that of the F_1 and, therefore, need not be repeated. No significant difference was found in either the tooth numbers or their dimensions.

Cx × *latidens* × *lucida.* The mouth parts of progeny from this mating (Tables II and III) are identical to those of Cx.

Ova size

The diameters of ova from *latidens, Cx,* and *lucida* females are distinctly different: 2.22, 1.81, and 1.53 mm., respectively (Table IV). Progeny of the Cx × *latidens* and Cx × *latidens* × *latidens* crosses had ova sizes identical to those of Cx. In spite of the fact that *latidens* has much larger ova, no increase in size resulted through hybridization with this species.

TABLE IV

Ova sizes (mm.) of progeny from various matings. Horizontal lines separate groups significantly different ($P < .05$) from each other

Mating	N	Mean
latidens × latidens	15	2.22 ± 0.03
lucida × lucida	15	1.53 ± 0.02
Cx × lucida	15	1.81 ± 0.08
Cx × latidens	15	1.84 ± 0.06
Cx × latidens × lucida	15	1.78 ± 0.08
Cx × latidens × latidens	15	1.77 ± 0.02

Inheritance of body pigment pattern

The most prominent feature of *P. latidens* is the pattern of black vertical bars and spots along the sides of the body. Spots, 8 to 11 per side, vary from 0.25 mm. to 1.25 mm. in diameter and are arranged along the midline. The bars are 0.5 to 1 mm. wide and extend from the back down the sides where they always pass through a spot and extend a short distance below the midline. The number of bars varies from one to five per side in *latidens* from the Mocorito, but three or four is by far the most common. In other rivers the mode may shift to either side of this. The first bar in practically all specimens is located just back of the pectoral fins. No other consistency is evident in the arrangement of spots and bars among individuals or even between the left and right sides. Bars may alternate with spots or as many as four bars may occur in succession uninterrupted by spots.

This pattern of bars and spots has been test mated (unpublished data) against the narrow barred pattern of *P. fasciata* (Meek), a species closely related to *latidens,* and found to have polygenic inheritance, in that there is a blending in the F_1 generation and a gradual restoration of the parental phenotype in the backcrosses.

P. lucida and Cx are uniformly olive color with no bars and spots. Mating combinations of *lucida* and Cx with the boldly marked *latidens* thus involve phenotypes of high contrast. When the plain or unmarked Cx was mated to the barred and spotted *latidens*, all 81 F_1 offspring had spots but no bars (Fig. 4). The spots of the hybrid were similar to those of *latidens* as regards size and position but were in general fewer in number, the range being one to 7 per side with no number favored over any other. The 6 males resulting from this mating exhibited the same spotted pattern found in the 81 females.

In the backcross to *latidens,* one might expect with normal segregation to find a few bars added to the spotted pattern or an elongation of the spots as was characteristic of the *latidens* × *fasciata* crosses; however, this was not the case, the pattern remained unaltered; the spotted condition of the 18 backcross progeny was identical to that of the F_1 generation. One might further expect that the Cx × *latidens* F_1 offspring mated to *lucida* would bring about a reduction in the number of spots or perhaps a dilution in the intensity of expression. The results, however, were more dramatic in that spots were totally lacking in the 8 progeny from this mating.

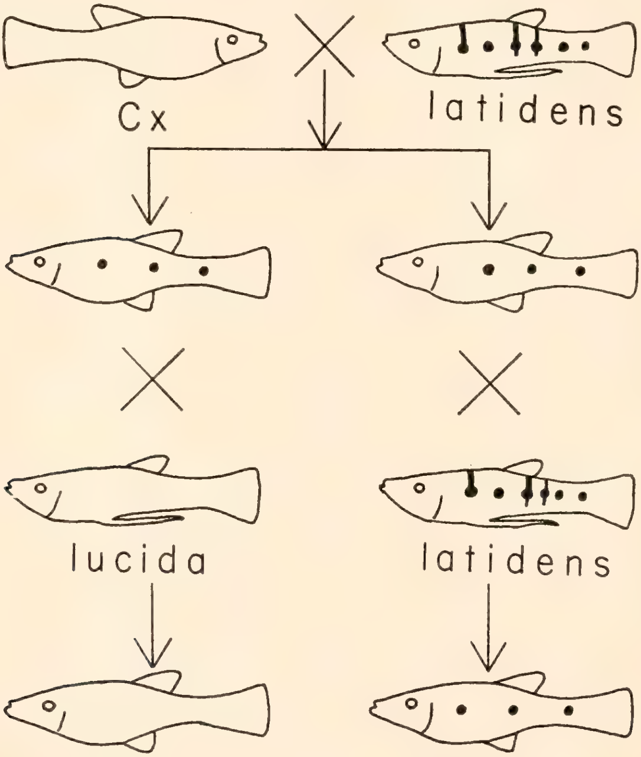

FIGURE 4. Inheritance of body pigment pattern in hybrid crosses.

DISCUSSION

Of the 14 species of *Poeciliopsis* that have been studied in the laboratory, 10 have produced fertile offspring when mated with the all-female form *Poeciliopsis* Cx (Miller and Schultz, unpublished). In all of these matings the offspring shared characteristics of both parents, and must, therefore, be considered true hybrids and not the fruit of parthenogenesis or gynogenesis. It is important from both an evolutionary and a systematic point of view to determine the impact that fertile hybrids such as these might have on a natural population. The question in point is whether or not characteristics of Cx can be incorporated into the bisexual population that provides the male, or whether the all-female population itself might undergo alterations as a result of continuously mating with other species.

In the Rio Mocorito, Cx is normally propagated by mating with males of *P. lucida,* a species so uniform morphologically as to offer no marker genes for tracing the paternal hereditary materials. This species, furthermore, is so similar to Cx that Miller (1960) referred to Cx as an all-female form of *lucida.* Detailed morphological analyses, however, reveal differences in the structure of the mouth, in the pigmentation (live specimens only) and in the structure of the genitalia, in the teeth, in the ova size, and in others, all of which remain consistent for Cx even after 10 generations of *lucida* matings. In view of this seemingly matroclinous mode of inheritance, the fate of the paternal chromosomes becomes the key to an understanding of the all-female mechanism. By selecting *P. latidens* as a mate for Cx, a great constellation of contrasting characters was made available, from which 6 were selected for detailed study in hybrid crosses with Cx. The following characteristics were found to differ markedly in all three forms, that is, Cx, *lucida,* and *latidens:* (1) genitalia; (2) lip shape; (3) structure of the dentary bones; (4) number, form, and size of the inner and outer teeth; (5) body pigments; and (6) ova size. Since expression of most of these characters, ova size being a possible exception, appears to be polygenic, marker genes, in a sense, are provided for a large portion if not all of the male's chromosomes.

The inheritance pattern of these characters in F_1 progeny of the Cx × *latidens* cross is by and large one of intermediacy; however, the form, count or measurement is usually somewhat closer to that of one parent than the other. The shape of the lips, the shape and size of the outer teeth, and the width of the inner teeth, for example, although intermediate, more nearly approach those of *latidens,* whereas the shape of the inner teeth and the ova size are the same as those of Cx. Judgment of bone shapes and pigment patterns is too subjective to enable more than a declaration of intermediacy. The inheritance patterns of the characters involved is not unexpected for traits influenced by multiple genes and the fact that they are not exactly intermediate might simply indicate unequal dosage effects from the different parents. The fact that the outer teeth in both the Cx × *latidens* cross and the F_1 × *latidens* backcross are fewer in number than in either parental form would at first seem contradictory to all that's reasonable. Once the composition and reproductive mechanisms of Cx are more adequately defined this matter will be accounted for. In any case, inheritance of the traits studied leaves no question that both parents contribute significantly to the genetic makeup of the F_1 offspring.

If inheritance now in the backcross matings is typical of what is true of other fish hybrids (Hubbs, 1955; Atz, 1962), one would expect the backcross progeny in the one direction to be more like *latidens* than the F_1, and in the other direction more like *lucida* but with some *latidens* traits persisting. This is not the case, however. When F_1 females were mated to *lucida* males, not a trace of *latidens* influence remained in the offspring—all were identical to Cx in every respect. It is apparent that none of the *latidens* chromosomes were transmitted through the eggs: All were lost, presumably during oogenesis. There, furthermore, is no evidence that crossing-over introduces male genes into the Cx line. This means, then, the eggs produced by the F_1 female that united with the *lucida* sperm were purely matroclinous. It would follow that in the backcross of the Cx × *latidens*, F_1 to *latidens* this egg, too, would contain only the genetic elements of the mother; hence, the phenotypic backcross in reality is genetically identical to the initial Cx × *latidens* mating. This supposition proves to be true by the fact that the backcross progeny were identical to the F_1 progeny—no increase in *latidens* traits took place.

We deal thus with an isolating mechanism that is complete. There is little likelihood that wild populations of Cx are genetically altered by either *lucida* or *latidens* but that the male genome is only borrowed for the production of each generation. It is furthermore unlikely that Cx elements are incorporated in the gene pools of either bisexual form.

The frequency with which Cx and *latidens* hybridize in nature has yet to be determined. The matter is complicated by the fact that numerous spotted forms found in the Mocorito resemble the Cx × *latidens* hybrid but are not identical and seem to be the result of another hybrid phenomenon not yet resolved. There is, however, no question that the Cx × *latidens* cross does take place. From 9 pregnant wild Cx females separated from males at the time of their capture and later isolated in aquaria, 69 young were produced which upon reaching maturity were typically Cx and were obviously sired by wild *lucida* males. One of these females, however, in addition to 6 Cx-like young, produced a spotted offspring characteristic of the Cx × *latidens* F_1. This Cx female evidently had mated with both *lucida* and *latidens* males and produced progeny by each in a single brood.

In view of what is happening here genetically, a rather knotty problem of nomenclature arises. Normally one might assign to Cx a Latin name such as *Poeciliopsis prostituere* and when it hybridizes with *latidens*, expand the name to form *P. prostituere-latidens*; however, Cx is already a hybrid, with *lucida* as one of the parents. Since all of the paternal chromosomes of each generation are lost it is only the female genome that combines with *lucida* on the one hand and *latidens* on the other. Not only is the naming of a genome unprecedented but added to this is the fact that we do not even know what this genome looks like since the only observable morphological expression is as a hybrid in combination with *lucida* and occasionally *latidens*.

The earlier view (Schultz, 1961) that this mysterious genome itself has had a hybrid origin seems no less likely. It may in fact explain why the Cx × *latidens* offspring have fewer outer teeth than any of the parental forms involved in the matings. If one of the species of the initial cross that resulted in the all-female genome had a low tooth count, then the genes for tooth expression carried by this genome would call for a low number of outer teeth. This genome, therefore, in combination with *lucida*, which has many teeth (29.07), results in Cx, a form with a moderate number of teeth (27.00). If the tooth count of the all-female genome is less than that of *latidens* (24.15), then in combination with this species, intermediate expression results in a tooth count lower (21.47) than either of the phenotypic parents (Cx or *latidens*).

If such a mechanism is operational as far as the outer teeth are concerned, there is no reason to believe that it is not also operational in the formation of other characters as well. The fact that many of the characters of the Cx × *latidens* F_1 progeny were not exactly intermediate between the parental forms, as Hubbs (1955) and others found in hybrids, but were closer to *latidens*, suggests that this all-female genome carries traits rather similar to *latidens*. The production of some Cx × *latidens* F_1 males (6.7%) indicates that the strength of the sex-determining genes of the all-female genome is somewhat more comparable to that of *latidens* than to that of *lucida* since all progeny from Cx × *lucida* matings are females. The implication here does not refer to a likelihood that *latidens* participated in the hybrid synthesis of the all-female genome. It seems more plausible that a species related to *latidens* was involved. It is further expected that when this species is found and mated to Cx, a breakdown of the all-female mechanism, complete with the production of fertile males, will occur along with the restoration of random assortment during meiosis.

Collections from specific localities in the Rio Mocorito contain, in addition to the above forms, *Poeciliopsis viriosa* Miller. The evidence from preserved material suggests that this species has not only hybridized with Cx in nature but that the F_1 progeny have mated with both *lucida* and *viriosa*, producing backcross progeny recognizable as such. Experimental studies directed toward a verification of the morphological determinations are currently in progress. It is possible that these studies will provide a key to the origin of the all-female genome and ultimately lead to its synthesis in the laboratory.

This study embodies many helpful suggestions of Dr. Robert R. Miller and Mr. Howard Huddle, both of whom assisted with the collecting.

SUMMARY

1. Hybridization experiments between an all-female fish, *Poeciliopsis* "Cx," and the males of two bisexual species, *P. lucida* and *P. latidens*, have provided genetic evidence that hereditary materials of both the mother and the father combine to form the F_1 progeny. The entire male genome, however, appears to be simply "on loan" for each generation. None of the male's chromosomes, apparently, are incorporated in the ova; thus, the integrity of the maternal line is maintained, even though the all-female form is forced to rely on sperm from other species to maintain itself.

2. This mode of reproduction provides an isolating mechanism as complete as that of parthenogenesis or gynogenesis.

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EFFECT OF HYPOPHYSECTOMY ON THE FUNCTION OF THE KIDNEY OF THE EURYHALINE TELEOST, FUNDULUS KANSAE[1]

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It now seems well established that hypophysectomy markedly reduces the ability of several euryhaline teleosts to survive in fresh water (Pickford *et al.,* 1965; Ball and Ensor, 1965; Handin *et al.,* 1964; Schreibman and Kallman, 1963, 1964; Ball, 1962; Pickford and Phillips, 1959; Pickford and Atz, 1957). The available data also suggest that such fishes are unable to maintain normal levels of blood-electrolytes in such an environment (Ball and Ensor, 1965; Leloup-Hatey, 1964; Burden, 1956), which leads to the supposition that the function of those organs most intimately concerned with salt and water metabolism, *i.e.,* the gills and kidney, has been affected by hypophysectomy. To date, few data have appeared to support such a supposition (Stanley and Fleming, 1963; Chester Jones *et al.,* 1965).

The renal physiology of the euryhaline teleost, *Fundulus kansae,* has been a subject of investigation in this laboratory for some time, and, during the course of these studies, several effects of hypophysectomy have been observed. We wish here to report the results of such studies.

Materials and Methods

Fundulus kansae was collected from a salt spring "Boonslick" located in Howard County, Missouri. The routine handling of the animals, the methods used to obtain urine and serum samples, to estimate urine volumes and glomerular filtration rates (GFR), and the analytical methods employed have all been described elsewhere (Fleming *et al.,* 1964; Stanley and Fleming, 1964; Fleming and Stanley, 1965) and need not be repeated here. Hypophysectomy was carried out *via* the opercular route recommended by Abramowitz (1937) under MS-222 (Sandoz) anesthesia. Controls were sham-operated in the same manner, except that the bone covering the pituitary was not pierced. With one exception noted below, the operation was performed on fresh-water-adapted fish which were returned to fresh water. Survival after hypophysectomy is good, providing that adequate calcium ion is made available to the animals (unpublished data).

The sea water used here was prepared from a commercial salt mix (Seven Seas Marine Mix, Utility Chemical Co., Patterson, New Jersey), and was brought to a concentration of 1000 mOsm/kg. All experiments were run at 19 ± 10° C.

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[1] Supported by a NSF Cooperative Fellowship to the senior author and by NSF Grant GB-2264.

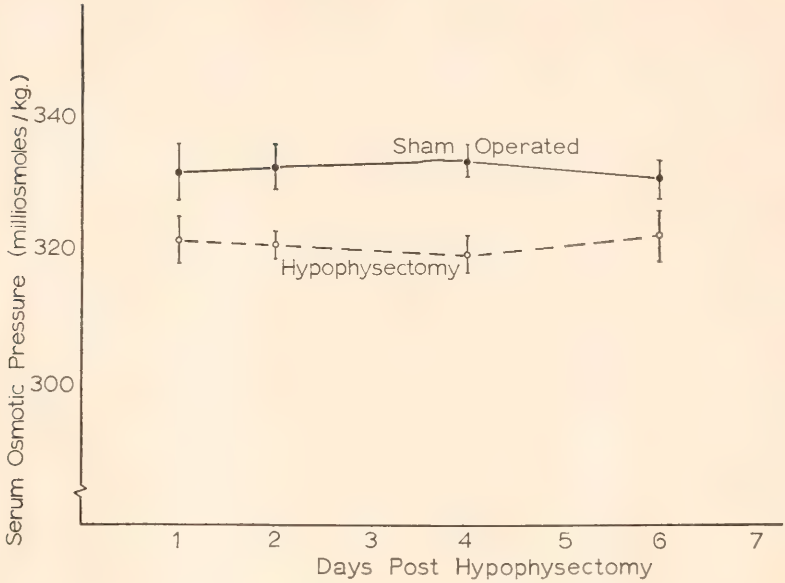

FIGURE 1. Effect of hypophysectomy on the serum osmotic pressure of the killifish, *F. kansae*, held in fresh water. Data show the mean ± S.E. for 7 animals at each point.

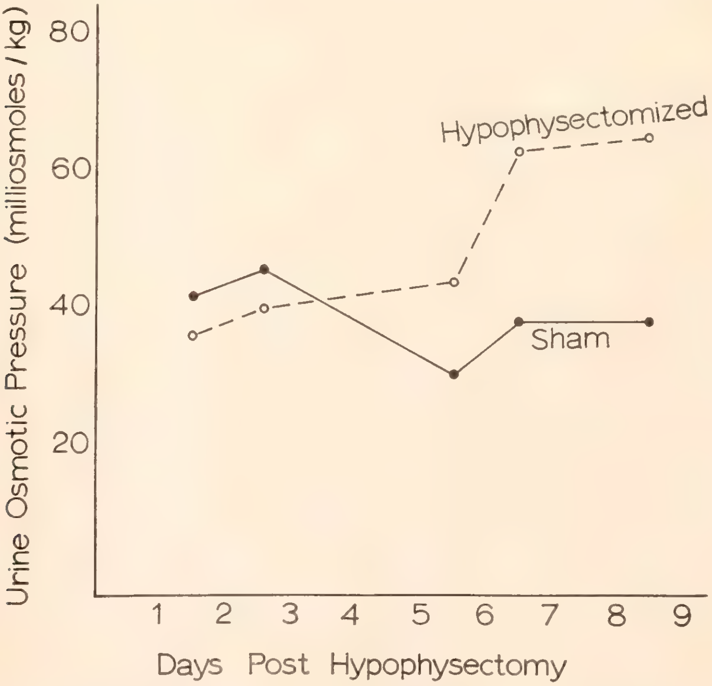

FIGURE 2. Effect of hypophysectomy on the osmotic pressure of the urine of animals in fresh water. Each point is the mean taken from three animals.

RESULTS

1. *Time sequence of responses to hypophysectomy*

The responses to hypophysectomy reported here do not appear simultaneously. As shown in Figure 1, a drop of the osmotic pressure of the serum of hypophysectomized fish is observed on the day after surgery. Since this response is seen only in hypophysectomized fish and is maintained indefinitely, it cannot be attributed to shock alone. In another experiment the serum osmotic pressure was 330 ± 3.6 milliosmoles (mean ± S.E.) for sham-operated animals and 312 ± 1.8 milliosmoles for hypophysectomized animals at 13 days post-operation.

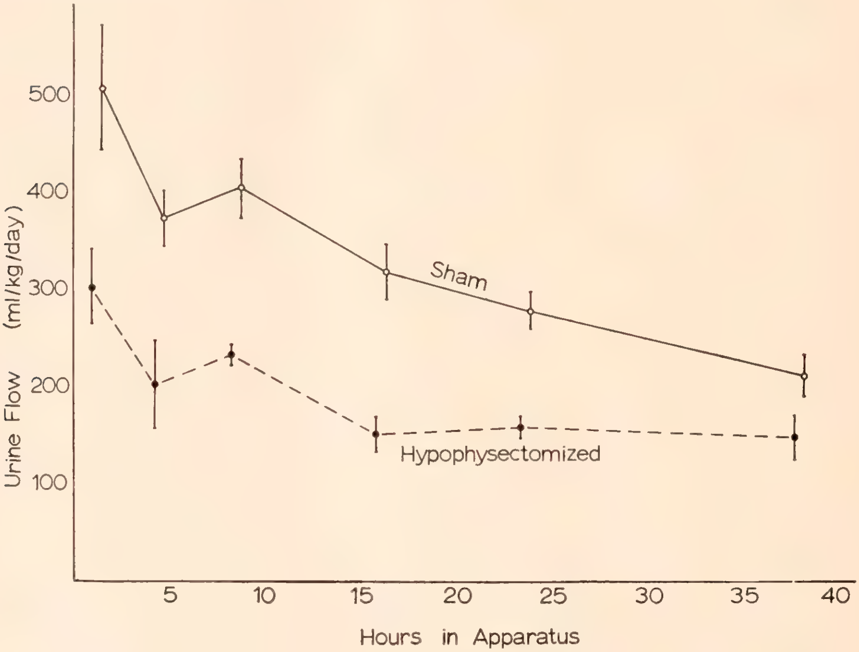

FIGURE 3. Comparison of urine flow of sham-operated and hypophysectomized *F. kansae* held in fresh water. Data show the mean flow ± S.E. for 7 animals.

A second response, a rise of the osmotic pressure of the urine (Fig. 2), does not appear until approximately one week after hypophysectomy. The appearance of still a third response, *i.e.,* a drop in the rate of urine excretion (Fig. 3), is somewhat variable, and may or may not be present at the time that the osmotic pressure of the urine starts to rise. It has been our practice to hold hypophysectomized animals for 12–14 days before use, in order to ensure that the above responses will all be present. As shown in Table I, the changes in the osmotic pressure of the urine and serum reflect changes in the concentration of the electrolytes found in these fluids. Urine sodium is elevated markedly, and urine potassium to a lesser extent. At the same time, serum sodium levels are reduced slightly. Total-body sodium is also less than that of the controls. No changes were noted in serum or total-body

TABLE I

Effect of hypophysectomy on urine and serum sodium and potassium, total body sodium and potassium, and total body water. The values given are the mean ± S.E. The number of animals in each sample is given in brackets. Urine data based on samples collected between the 21st and 26th hours after cannulation

	Sodium (meq/l.)	Potassium (meq/l.)	Water content (%)
Urine			
Hypophysectomized	47.8 ± 7.6 (11)	1.06 ± 0.20 (11)	
Sham-operated	15.1 ± 1.1 (10)*	0.45 ± 0.10 (10)*	
Serum			
Hypophysectomized	145.9 ± 2.5 (9)	4.17 ± 0.42 (11)	
Sham-operated	157.8 ± 1.3 (13)*	3.80 ± 0.29 (11)	
Total Body	(meq/kg.)	(meq/kg.)	
Hypophysectomized	55.2 ± 2.1 (18)	64.8 ± 1.02 (12)	74.88 ± 0.34 (12)
Sham-operated	62.5 ± 1.0 (12)*	65.4 ± 1.63 (18)	74.62 ± 0.29 (12)

* $P = 0.05$.

potassium levels, or in the percentage of body water. The observed reduction in urine flow may serve to reduce renal sodium loss somewhat but is not entirely effective. Thus, as shown in Table II, hypophysectomized animals lost, over a 45-hour period, over twice as much sodium *via* the kidney as did their controls.

2. *The diuretic response*

An examination of Figures 3, 5, and 6 shows a characteristic response of control animals to handling, *i.e.*, a marked diuresis of variable duration. Examination of the data for the controls reported in Figure 4 indicates that the diuretic phase is due, in part, to reduced tubular reabsorption of water, *i.e.*, the urine/serum C^{14} inulin ratio is less than 2 for several hours after cannulation. In other experiments, we have measured U/S ratios close to unity during the diuretic peak. Figure 5 shows that the diuretic response of the controls is also due, in part, to an elevated GFR. The same data show that while hypophysectomized animals may show some initial diuresis, the response is markedly reduced. The data re-

TABLE II

Renal sodium loss of hypophysectomized and control animals over a 45-hour period. Each value is the mean ± S.E. measured from a sample of 7 animals

	μeq. Urinary Na⁺/gm./hr.	
Hours	Hypophysectomized	Controls
0–3	0.99 ± 0.11	0.70 ± 0.12
3–11	0.79 ± 0.11	0.36 ± 0.06
11–21	0.49 ± 0.15	0.16 ± 0.01
21–31	0.56 ± 0.12	0.20 ± 0.03
31–45	0.53 ± 0.09	0.16 ± 0.02
Total Na^+ loss	27.21 ± 2.75	10.82 ± 0.50

ported in Figure 4 indicate that hypophysectomized fish do not reduce tubular reabsorption of water during the diuretic period. However, hypophysectomized animals also do not show an elevated GFR as seen in controls during the diuretic period (Fig. 5).

3. *Effects of a sudden change of environment*

Hypophysectomy also affects the renal responses shown by *F. kansae* when transferred suddenly from a hypotonic to a hypertonic environment. Figure 6 compares the rates of urine flow of hypophysectomized and control animals that were switched suddenly from fresh water to sea water. Examination of Figure 6 shows that the control animals were able to reduce urine flow rapidly, and had established a new and steady rate of urine flow after being in sea water for 5 or 6 hours. Hypophysectomized animals also reduce urine flow, but do so at a much slower rate. Thus, in the experiment reported in Figure 6, these fish required almost 40 hours to reach levels that their controls had achieved by four hours post-transfer.

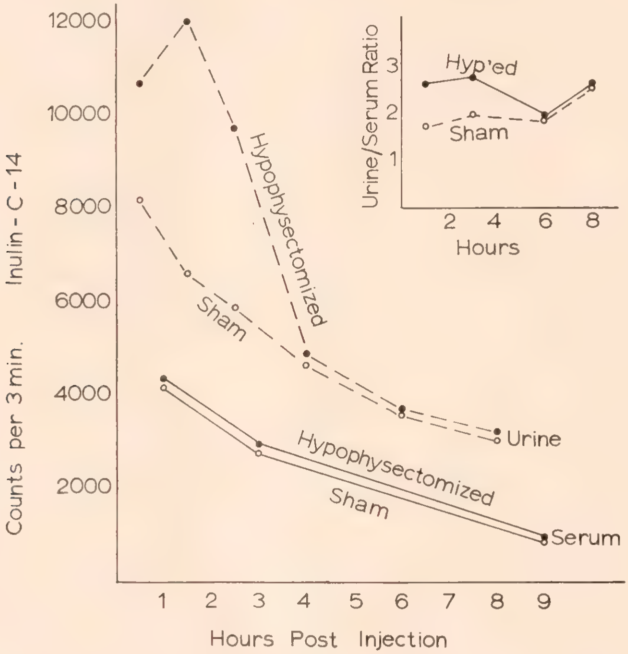

FIGURE 4. Rate of clearance of C^{14} inulin from the serum and its appearance in the urine of fish in fresh water. Serum and urine data at each point are the means of three determinations.

Figure 7 reports the changes in the osmotic pressure of the urine measured from the same experiment. Inspection of Figure 7 shows that the osmotic pressure of the urine taken from hypophysectomized animals in fresh water was almost triple that of their controls. However, the situation was quickly reversed after

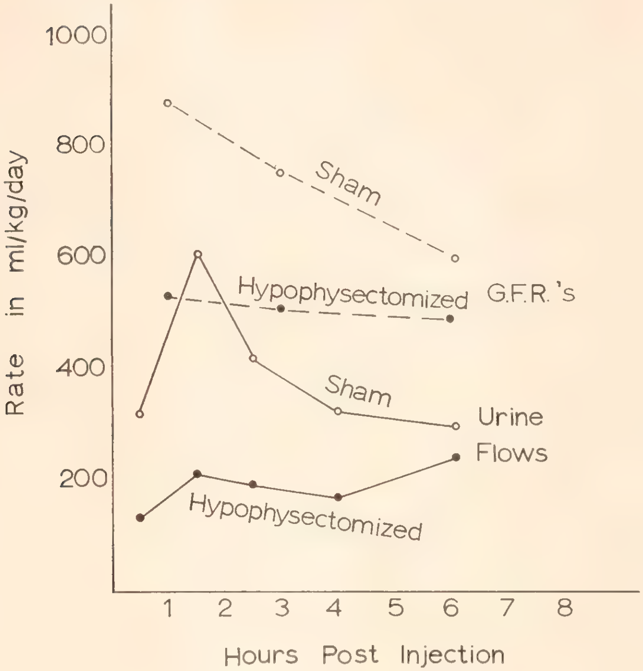

FIGURE 5. Glomerular filtration rates of sham-operated and hypophysectomized *F. kansae* in fresh water. GFR's calculated from the data in Figure 4 and the urine flow.

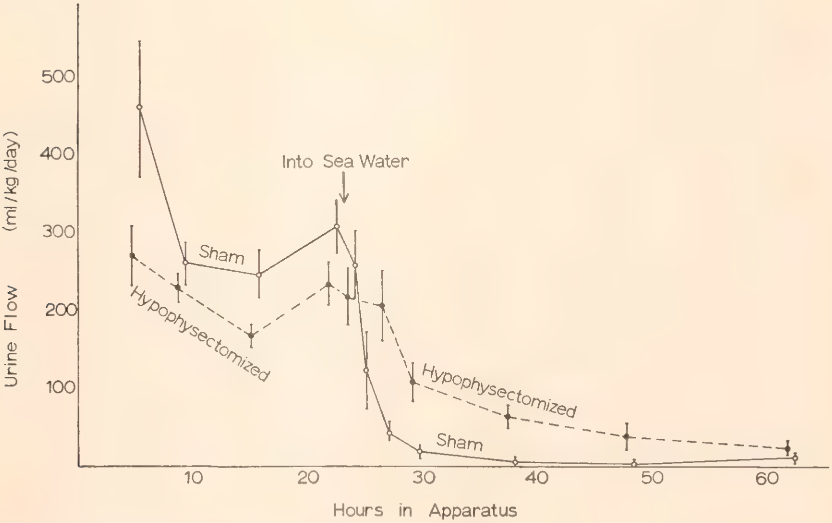

FIGURE 6. Effect of a sudden transfer from fresh water to sea water on urine production. Each point shows mean ± S.E. for 7 animals.

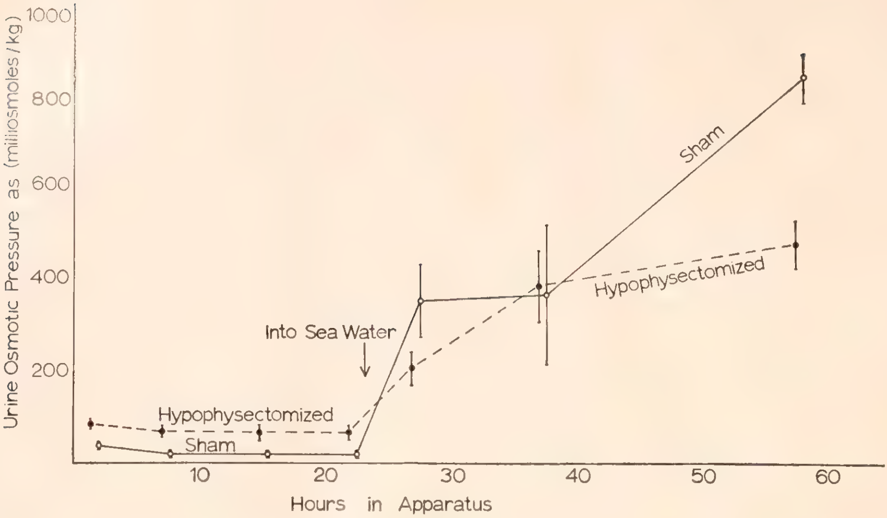

FIGURE 7. Changes in the osmotic pressure of the urine which follow transfer from fresh water to sea water. Data collected from the same experiment shown in Figure 6.

switching the environment to sea water. Further, as shown in Table III, the osmotic pressure of urine taken from the control animals at the termination of the experiment was hypertonic to the serum, while that of the hypophysectomized animals was hypotonic. The ability of *F. kansae* to excrete a blood-hypertonic urine has been reported previously (Stanley and Fleming, 1964; Fleming and Stanley, 1965).

4. *Pre-adaptation and serum osmotic pressure*

During the course of other experiments, it was noted that a short exposure to saline would markedly alter the course of adaptation to sea water, at least as judged by the magnitude of the changes in the osmotic pressure of the serum which follow a transfer from fresh water to sea water. Figure 8 reports the effect of a 9-hour exposure to 40% sea water on the changes in serum osmotic pressure

TABLE III

Comparison of the osmotic pressure of the serum and urine of control and hypophysectomized animals. Data taken at the termination of the experiment shown in Figures 6 and 7. The lower number of urine samples is due to the failure of some animals to excrete sufficient urine for a measurement. Osmotic pressures in mOsm./kg.

	Control			Hypophysectomized		
	N	$\bar{x}$	±S.E.	N	$\bar{x}$	±S.E.
Serum	5	326	11	6	313	20
Urine	3	455	27	4	256	26

which followed such a transfer. In this experiment the routine followed was 9 hours in 40% sea water, 18 hours in fresh water, and then a transfer into 100% sea water. As shown in Figure 8, 18 hours in fresh water was sufficient time for the osmotic pressure of the serum to fall to levels identical to that of controls that had been held in fresh water continuously. Figure 8 also shows that the short exposure to dilute sea water markedly changed the response to the challenge presented by full-strength sea water.

As shown in Figure 9, hypophysectomy alters the response seen when preadapted animals are transferred from one environment to the other. In this experiment, a large number of fish (160) were held in sea water for several weeks. After this adjustment period, half the animals were hypophysectomized and returned to sea water. Five days later all the fish were transferred to fresh water for 48 hours, sufficient time for the osmotic pressure of the serum of the hypophysectomized fish to fall below that of their controls. The fish were then transferred back into sea water and the osmotic pressure of the serum followed for 23 hours. Then the remaining fish were returned to fresh water. Figure 9 shows that the serum osmotic pressure of the hypophysectomized fish rose above that of their controls after the first transfer, and, for that matter, appeared to be rising slowly

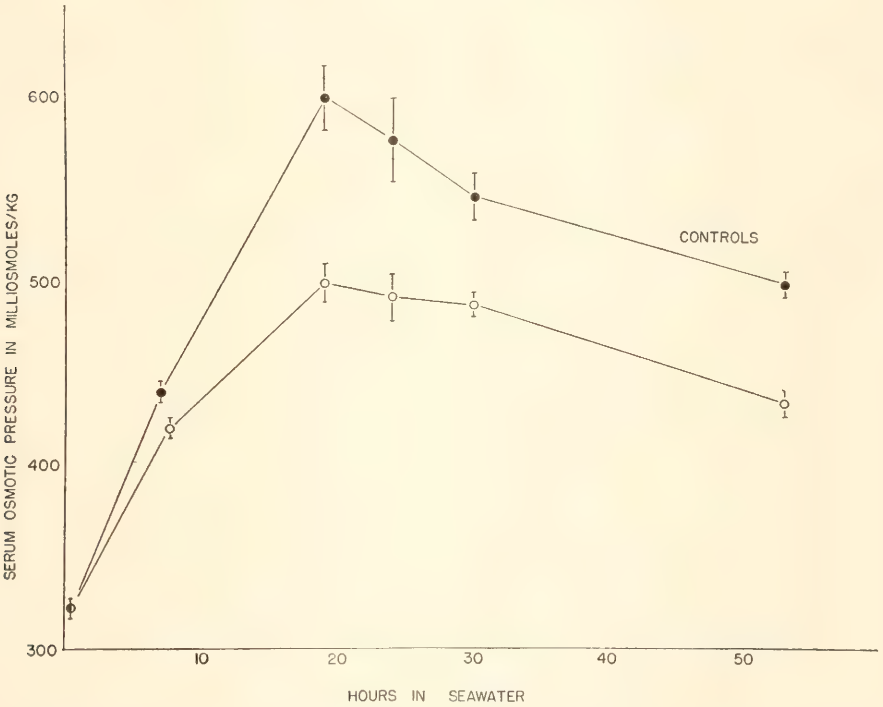

FIGURE 8. The effect of a 9-hour exposure to 40% sea water on the ability of *F. kansae* to adjust to a transfer from fresh water to sea water. Each point shows the mean ± S.E. for 7 animals.

long after the controls had reached a fairly steady plateau. The hypophysectomized animals do show some signs of their past history, however, for the peak values reached were still well below those shown by still another control group which had not been exposed to saline before being transferred into sea water.

Neither group had any difficulty in readjusting to fresh water, although, once again, the time course and degree of adjustment was somewhat different in the two groups. Thus, by $8\frac{1}{2}$ hours after the return to fresh water, the serum osmotic pressure of the control animals was significantly lower ($P = 0.05$) than that measured at the start of the experiment. Then the osmotic pressure rose to the original levels. The serum osmotic pressure of the hypophysectomized animals dropped precipitately immediately after the return to fresh water ($1\frac{1}{2}$ hours) and then fell slowly towards the original values, although these had not been reached at the time the experiment was terminated.

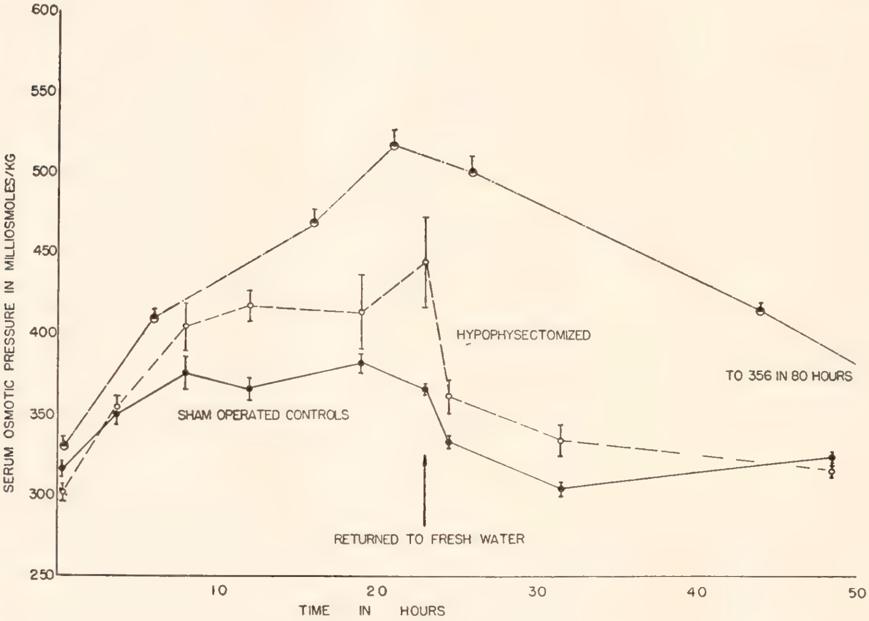

FIGURE 9. Effect of hypophysectomy and pre-adaptation to sea water on the ability of *F. kansae* to adjust to a sudden transfer from fresh water to sea water. The hypophysectomized animals and their controls had been adapted to sea water for several weeks and then returned to fresh water for 48 hours. The other group of controls (top line) had not been exposed to saline. Each point shows the mean ± S.E. for 6 animals.

DISCUSSION

It is clear that *F. kansae* can survive in fresh water after hypophysectomy. In this regard the plains killifish more closely resembles the European eel (*Anguilla anguilla* L.) which also survives well after such surgery (Fontaine *et al.*, 1949; Chester Jones *et al.*, 1965), than it does a number of other euryhaline teleosts including the closely related *F. heteroclitus* (Pickford *et al.,* 1965). There are, however, clear-cut differences between this killifish and the eel, for the latter survives well in distilled water—even after hypophysectomy (Chester Jones *et al.*, 1965). *F. kansae,* on the other hand, will not tolerate such an environment for more than a few days, at best.

While the plains killifish can survive in fresh water after hypophysectomy, removal of the pituitary does alter the osmoregulatory patterns of the animal. The kidney is one of the target organs affected, as shown by a drop in volume and a rise in osmotic pressure of the excreted urine. A preliminary report (Chester Jones *et al.,* 1965) indicates that the eel shows a similar response for urine volumes, but in this case, there was a reduction in urinary sodium loss.

Since *F. kansae* survives well in fresh water after hypophysectomy, it seems clear that the accelerated loss of sodium *via* the kidney does not present an insurmountable challenge to the animals. Nevertheless, the changes seen are not trivial and raise a number of questions. Thus it becomes useful to learn if the reduction in urine flow is due to (1) a reduction in GFR, or (2) an increase in the tubular reabsorption of water.

On the surface, at least, the first alternative would seem to offer the simplest explanation of our data, and would only require as a corollary that the tubular reabsorption of ions be reduced after hypophysectomy. A large body of literature exists to provide precedent for such responses.

There are, however, a number of difficulties. Thus, if one considers that a reduction of GFR has followed hypophysectomy, it then becomes necessary to suppose that these animals must either evoke another route of water excretion in order to handle the ever-impinging water load, or effectively reduce the net influx of water across permeable surfaces.

A second difficulty lies in the report of Holmes and McBean (1963), who found that corticosterone markedly reduced the GFR of rainbow trout (*Salmo gairdneri*) held in fresh water. The implication is that glomerular filtration rates are, at least in part, under the control of the pituitary-interrenal axis. Our results are not inconsistent with such an implication, but would require that such control would serve to increase the GFR, rather than to reduce it. Unfortunately, Holmes and McBean (1963) did not use hypophysectomized animals, and the dose of steroid administered (5 mg.) seems to be beyond the physiological range.

A third difficulty lies in the fact that, to date, we have not been able to show any difference between the filtration rates of hypophysectomized and sham-operated controls, except during the diuresis which follows handling. This point is still under investigation and must remain open.

The second alternative, *i.e.*, an increase in the tubular reabsorption of water, differs from the first in that it does not require as marked a change in the pattern of ion reabsorption. It would seem to require, however, an even more drastic solution to the problem of the impinging water load, for an increase in water reabsorption would hardly be a positive contribution to the necessity of eliminating excess water—unless one supposes that water permeability is controlled by the rate of urine excretion, instead of *vice versa.* A decision between the two alternatives (if indeed the situation is that simple) cannot be made until the question of filtration rates is closed.

It may well be that the situation is not so simple, and, in this regard, Chester Jones *et al.* (1965) have recently put forward a hypothesis that would, if demonstrated, complicate the situation considerably. They suggest that in the eel, urine has a double original, *i.e.*, from the glomerular filtrate and from the addition of water to the filtrate *via* the peritubular capillaries of the renal portal system. This hypothesis was advanced to explain the paradoxical effects of hormones on the renal function of the eel. We have not considered the possibility of such a mechanism operating in *F. kansae* nor have we studied the effects of neurohypophysial hormones on renal function, but we can point out that mammalian ACTH does not affect the rate of urine excretion of hypophysectomized killifish (Stanley and Fleming, 1963).

It is well known that teleosts are slow to show responses to hypophysectomy, at least as judged by histological criteria (Handin *et al.*, 1964; Pickford and Atz, 1957). Our data show that some physiological responses appear in a relatively short period after surgery, a result also found by Ball and Ensor (1965) and Chester Jones *et al.* (1965). Thus it appears that pituitary control may, in some cases at least, be more precise than that suggested from histological studies.

The data presented in Figure 9 suggest that the pituitary may be more involved in the adjustments required of *F. kansae* when presented with a blood-hypertonic challenge than when the opposite situation obtains. Such results are consistent with a number of studies which demonstrate that the interrenals often show signs of hyperactivity when such transfers are carried out (Olivereau, 1962).

SUMMARY

Hypophysectomy has been shown to affect the pattern of urine formation and excretion by the euryhaline killifish, *Fundulus kansae*. Changes noted in fresh water include (a) a drop in urine volume, (b) a rise in osmotic pressure, due in part to increased concentrations of sodium and potassium in the urine, and (c) a marked increase in the time required to shut off urine flow when transferred suddenly from fresh water to sea water. Hypophysectomized animals in fresh water also show a slight but consistent reduction in the osmotic pressure of the serum. Pre-exposure to saline increases the ability of hypophysectomized animals to respond to an osmotic challenge, but they do not respond as well as their controls.

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RNA, PROTEIN AND URIC ACID CONTENT OF BODY TISSUES OF PERIPLANETA AMERICANA (L.) AS INFLUENCED BY CORPORA ALLATA DURING OVARIAN DEVELOPMENT

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Thomas and Nation (1966) showed that allatectomy results in a decrease in total haemolymph protein concentration and, more specifically, in decrease of one particular kind of protein in *Periplaneta americana* (L.) females.

L'Hélias (1953a, 1953b) observed that the corpus allatum hormone favors synthesis of proteins and RNA in *Carausius morosus*. Vanderberg (1963) more recently confirmed a positive correlation between the allatum hormone and active synthesis of RNA and protein in *Rhodnius prolixus* Stål.

These results prompted the more general study of the effects of allatectomy in *P. americana* females on total body protein, total body RNA, and of the ability of certain tissues to incorporate H^3-labelled amino acids. A determination of total body uric acid was also made following allatectomy since uric acid is one end-product of protein catabolism.

MATERIALS AND METHODS

Periplaneta americana females used in this study were maintained in the laboratory on dog biscuits and water. Allatectomy was performed according to Bodenstein (1953) no later than 48 hours after imaginal molt. Sham-operated insects served as controls. Post-operative maintenance of insects has been described (Thomas and Nation, 1966).

Preparation of samples

Body proteins, uric acid and RNA were determined on tissue homogenates of roaches from which haemolymph had been collected by centrifugation. The wings, legs, and alimentary canal of the roaches were removed, and the weighed insects were ground with sand and 10.0 ml. of ice-cold distilled water in a chilled mortar. The homogenate was filtered through cheesecloth and the filtrate used for protein, RNA and uric acid determinations.

The filtered homogenate was unsuitable for direct colorimetric determinations; hence the following procedure was employed to remove the undesirable contaminants that interfered with the determinations. One milliliter of the above filtrate was diluted to 10.0 ml. with water, and, with thorough mixing, 2.0 ml. each of cold 10% sodium tungstate and $\frac{2}{3}$ N H_2SO_4 were added and the mixture kept in an ice bath for about an hour to facilitate complete precipitation of proteins. The mixture was centrifuged in a Servall refrigerated centrifuge at 5900 *g* for 15 minutes. The clear supernatant containing the uric acid was stored for uric acid determinations. The precipitate was redissolved in 1.0 ml. of cold 10% sodium tungstate and diluted to 10.0 ml. with cold water. Protein was again precipitated by adding 1.0 ml. of $\frac{2}{3}$ N H_2SO_4. The mixture was centrifuged again after 15 minutes. The precipitate was dissolved and the precipitation procedure repeated once more to bring all uric acid from the precipitate into solution. The precipitate was finally defatted by suspending it in a solvent mixture of cold 95% ethanol, chloroform, and ether (1:2:2). The suspension was centrifuged, the protein pellet resuspended, and the process repeated once more. The defatted protein pellet was brought into solution by adding a few drops of 1.0 N NaOH solution and the volume made up to 10.0 ml. with cold water. A sample of 0.5 ml. was taken for protein determination. Protein was determined according to the method of Lowry *et al.* (1951). No uric acid could be detected in a 2.0-ml. sample with the uric acid reagent of Benedict (1922).

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The supernatants left after each protein precipitation procedure were pooled, and a sample of 0.5 ml. was assayed for uric acid by the colorimetric method of Benedict (1922), using the uric acid standard of Folin (1930), and the urea-cyanide reagent of Christman and Ravwitch (1932).

A sample of 5.0 ml. from the original tissue homogenate filtrate was employed for the determination of total RNA. This volume was diluted to 9.0 ml. with ice-cold distilled water, and 1.0 ml. of cold TCA solution having a concentration of 100 g./100 ml. was added to give a final concentration of 10% TCA. The solutions were thoroughly mixed and allowed to stand in the cold for an hour to insure complete precipitation. The mixture was later centrifuged in a refrigerated centrifuge at 12,000 *g* for 15 minutes. The precipitate was suspended in 9.0 ml. of cold distilled water and then precipitated with 1.0 ml. of 100% TCA. The precipitation procedure was necessary to remove all interfering substances. The precipitate was then defatted twice by suspending in a solvent mixture of cold 95% ethanol, chloroform and ether (1:2:2). This mixture was centrifuged and the supernatant discarded.

The defatted precipitate was then washed twice with ice-cold 5% TCA to remove the defatting solvent. RNA was extracted from the defatted pellet with 2.0 ml. of 5% TCA and heated for 15 minutes in a water bath maintained at 90° C. The mixture was cooled, centrifuged at 12,000 *g* for 15 minutes, and the clear supernatant assayed for RNA. The concentration of RNA was measured by the phloroglucinol reaction for ribose according to the method of von Euler and Hahn (1946). After color development, samples were read in a Coleman Junior Spectrophotometer at 620 mμ against a 5% TCA blank and a thymus RNA standard.

Radioisotope incorporation experiments

A sample of 14 allatectomized and 14 sham-operated females were injected through the femora of the metathoracic legs with 5 μc. of H^3-labelled glycine, leucine, and histidine mixture (specific acivity 2.5 mc./mM) dissolved in 0.1 ml. of physiological saline. At intervals of 1.5, 3, 6, 12, 24, and 48 hours after injection, fat body tissue, ovaries and midgut were removed and washed in several changes of saline to insure complete removal of non-incorporated amino acids. Tissues were

TABLE I

Comparison of body protein concentrations of allatectomized and sham-operated control females of the American cockroach, Periplaneta americana

Days after operation*	Allatectomized		Sham-operated control	
	Number of females	Protein concentration (g./100 g. body weight) $\bar{X}$ ± SD	Number of females	Protein concentration (g./100 g. body weight) $\bar{X}$ ± SD
7**	4	0.637 ± 0.08	5	0.78 ± 0.12
14	5	0.695 ± 0.07	4	0.94 ± 0.10
21	4	0.71 ± 0.06	4	1.06 ± 0.06
28	5	0.65 ± 0.06	5	0.897 ± 0.06
35	6	0.56 ± 0.08	5	0.88 ± 0.10

* All animals operated upon 8–48 hours after imaginal molt.

** Four newly molted females had an average concentration of 0.38 g./100 g. body weight.

dried in a desiccator, weighed, and converted to a uniform condition for counting as already described (Thomas and Nation, 1966). Samples were counted on a transistorized Tricarb Liquid Scintillation Counter (Packard Instrument Co.).

RESULTS

Effects of allatectomy on body protein concentration

The concentration of body proteins in the allatectomized and the sham-operated female roaches is shown in Table I. The concentration of body proteins in the allatectomized females is much lower than that of the sham-operated females of the same age.

The concentration of RNA in the allatectomized and sham-operated female roaches, calculated on a protein basis, is shown in Table II. The concentration of RNA in the allatectomized roaches 14 days after allatectomy was only 14.18 ±

TABLE II

Comparison of RNA concentration in the body tissues of allatectomized and sham-operated control females of the american cockroach, Periplaneta americana

Days after operation*	Allatectomized			Sham-operated control		
	Number of females	RNA concentration (mg./100 mg. protein) $\bar{X}$ ± S.D.	RNA concentration (mg./100 gm. body weight) $\bar{X}$ ± S.D.	Number of females	RNA concentration (mg./100 mg. protein) $\bar{X}$ ± S.D.	RNA concentration (mg./g. body weight)
7**	3	13.96 ± 0.65	96.5 ± 5.72	3	16.99 ± 2.96	185.0 ± 8.40
14	4	14.18 ± 0.69	106.0 ± 8.62	4	22.42 ± 3.10	265.7 ± 6.80
21	3	14.46 ± 1.64	97.6 ± 10.30	3	19.21 ± 3.59	254.6 ± 15.80
28	3	14.59 ± 1.46	89.9 ± 6.40	4	18.24 ± 3.85	214.0 ± 9.90

* All insects operated upon 8–48 hours after imaginal molt.

** Three newly molted females had an average concentration of 10.39 ± 0.82 mg./100 mg. protein and 91.90 ± 6.02 mg./100 g. body weight.

Table III

Comparison of uric acid concentration in the body tissues of allatectomized and sham-operated control females of the american cockroach, Periplaneta americana

Days after operation*	Allatectomized		Sham-operated	
	Number of females	Uric acid concentration (g./100 g. body weight) $\bar{X} \pm$ SD	Number of females	Uric acid concentration (g./100 g. body weight) $\bar{X} \pm$ SD
7**	5	4.49 ± 0.19	5	6.13 ± 1.41
14	3	4.47 ± 0.21	3	8.55 ± 1.69
21	4	4.36 ± 0.32	4	8.09 ± 1.16
28	4	4.14 ± 0.22	4	9.06 ± 0.45
35	4	3.96 ± 0.17	4	8.78 ± 0.97

* All insects operated upon 8–48 hours after imaginal molt.

** Four newly molted females had an average concentration of 4.18 g. ± 0.12 g./100 g. body weight.

0.69 mg./100 mg. protein, compared to 22.42 ± 3.10 mg. in the sham-operated females.

Table III shows the concentration of uric acid in the body tissues of allatectomized and sham-operated roaches. It is interesting to observe that in allatectomized females, the uric acid concentration remained approximately constant, while

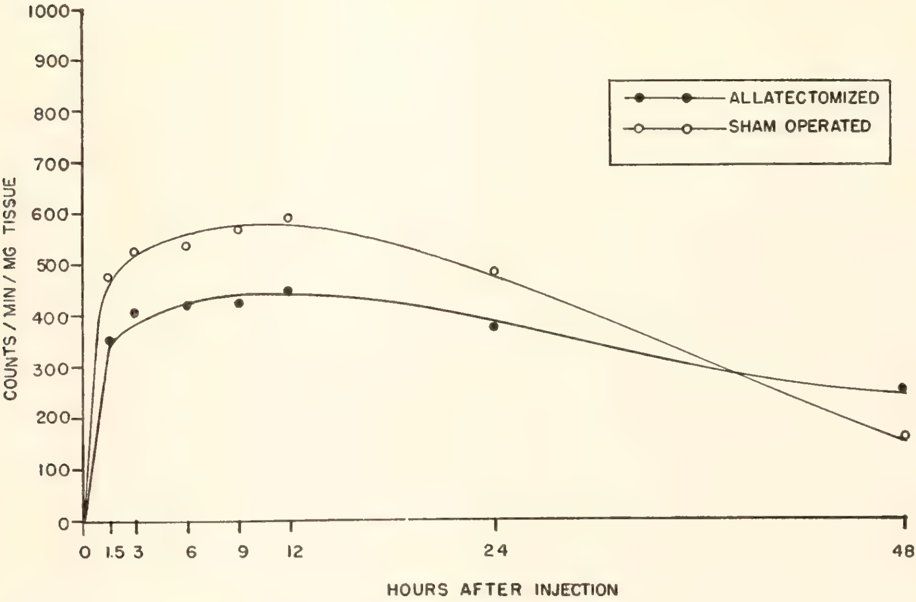

Figure 1. Rate of incorporation of tritium-labelled glycine, leucine, and histidine into the fat body of allatectomized and sham-allatectomized females of *P. americana* roaches (5 μc./roach). Points are averages of two experiments.

in the controls, it increased to more than double the concentration observed in the allatectomized females. However, five different analyses of fecal pellets of allatectomized and sham-operated roaches for uric acid, using the method of Wharton and Wharton (1960), failed to show any significant differences in excretion of uric acid. The lower concentration in allatectomized females is evidently the result of decreased protein catabolism.

Effects of allatectomy on the rate of incorporation of H^3-labelled amino acids

Figure 1 shows the sham-operated control females to have a greater rate of isotope incorporation and a greater total incorporation of label into fat body tissue than allatectomized females.

In allatectomized roaches, the uptake of tritiated amino acids by the ovaries was very slow compared to a very pronounced uptake by the ovaries of sham-operated females (Fig. 2). The fact that after 12 hours the specific activity in sham-operated controls was three times as great as the allatectomized females indicates marked inhibition of protein synthesis in ovaries of allatectomized females.

Incorporation of the label into midgut tissue of allatectomized and sham-operated females differed from the pattern shown in fat body and ovarian tissue, in that the total isotope incorporation was greater in allatectomized females than in control females (Fig. 3). Statistical analysis of the data (F test) showed

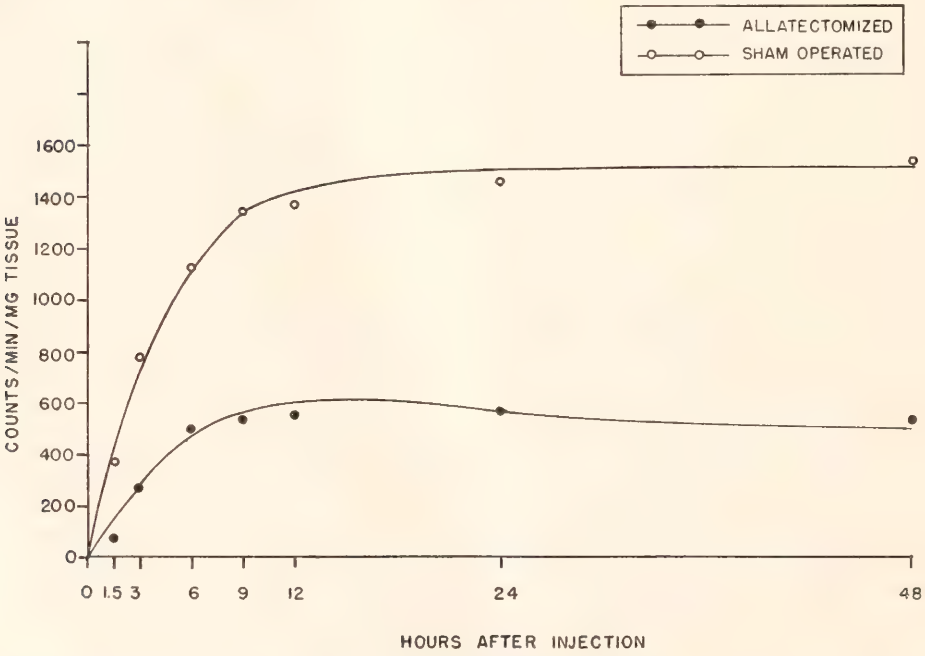

FIGURE 2. Rate of incorporation of tritium-labelled glycine, leucine, and histidine into ovarian tissue of allatectomized and sham-allatectomized females of *P. americana* roaches (5 μc./roach). Points are averages of two experiments.

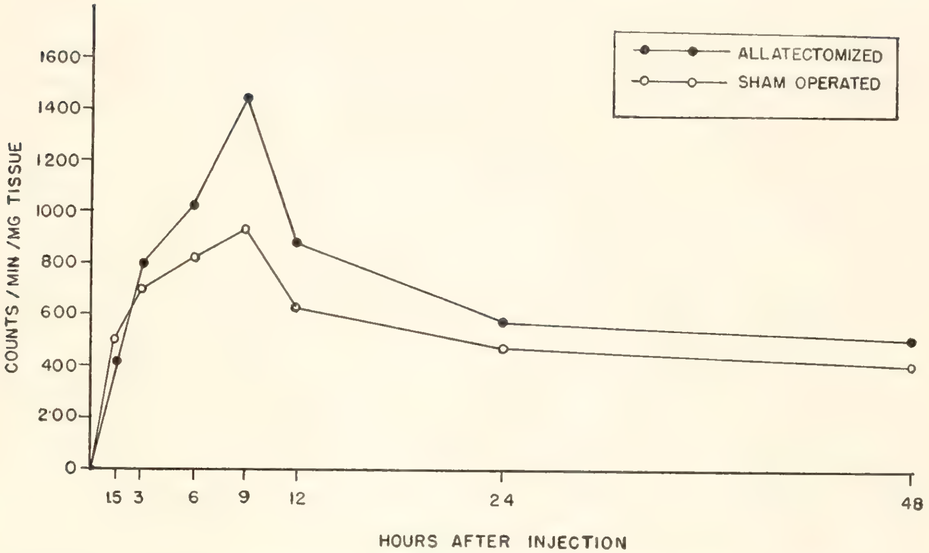

FIGURE 3. Rate of incorporation of tritium-labelled glycine, leucine, and histidine into the midgut tissue of allatectomized and sham-allatectomized females of *P. americana* roaches (5 μc./roach). Points are averages of two experiments.

that the difference is significant at 9 hours and 12 hours after injection of the radioactive amino acids.

DISCUSSION

We have shown that allatectomy during an ovarian cycle in *P. americana* females results in a lower level of total body RNA and protein, and that total body uric acid fails to increase as it does in control females. Furthermore, there is marked decrease in total incorporation of radioactive amino acids into protein of ovarian and fat body tissues following allatectomy.

The decreased body levels of protein and decreased synthesis of tissue proteins may be a consequence of a decrease in RNA synthesis. Clever and Karlson (1960) observed in larvae of *Chironomus tentans* that injected ecdysone induces chromosomal puffs, which could be demonstrated by autoradiography to be sites of RNA synthesis. It is possible that the allatum hormone functions in a similar manner to control RNA synthesis.

Uric acid in insects is formed principally from protein catabolism and the lack of an increase in urate level in allatectomized females, in the absence of any detectable increase in urate excretion, may be interpreted as the consequence of a decreased protein catabolism. Bodenstein (1953) observed a loss of urates from the fat body when corpora cardiaca were removed, but little or no effect was found upon removal of only the corpora allata. Our results support his observations that allatectomy has no influence on stored urates.

The most conclusive evidence for participation of the corpora allata in protein synthesis comes from the isotope incorporation experiments. In allatectomized roaches fat body tissue, ovarian tissue, and haemolymph (Thomas and Nation, 1966) showed marked inhibition in rate of amino acid incorporation and total amount of isotope incorporated into protein. A decrease in intracellular storage of proteins was also shown by fat body tissue stained for proteins according to Mazia *et al.* (1953).

In only one tissue examined, midgut tissue, was this pattern of inhibition not found; whether the greater incorporation of amino acids into midgut tissue of allatectomized females is the result of active flushing of the amino acids into the gut or an initial stimulation of protein synthesis cannot be ascertained from our experiments. The midgut is known in some insects to be active in protein synthesis and the inhibition of protein synthesis in haemolymph, ovary, and fat body may enlarge the free amino acid pool and promote synthesis in midgut tissue. It is conceivable that the allatum hormone controls protein synthesis in only some tissues, perhaps having little or no effect on midgut tissue.

The lower concentrations of body proteins, RNA, and uric acid, and the decreased rate of amino acid incorporation in allatectomized females when compared with sham-operated females, support the view that the corpus allatum hormone influences protein metabolism. Not only is general body protein decreased in allatectomized females but there are changes in certain proteins in the haemolymph (Thomas and Nation, 1966). The positive correlation of these changes in protein and RNA metabolism with the gonadal cycle in female *P. americana* is consistent with the view held by some workers that the allatum hormone controls ovarian and egg development through control of protein metabolism.

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SUMMARY

1. The concentrations of RNA, proteins, and uric acid in the body tissues of allatectomized and sham-operated female roaches were measured at intervals after the imaginal molt. The concentrations of RNA and proteins were substantially lower in allatectomized females when compared with sham-operated females. Uric acid concentration remained approximately constant in allatectomized females, while in the controls it increased to more than double the concentration observed in the allatectomized females.

2. The rate of incorporation of tritium-labelled amino acids into the fat body of allatectomized females was slow compared to that of the sham-operated roaches. A very slow rate of incorporation into ovarian tissue was observed in allatectomized females, as against a rapid rate of incorporation in the controls.

3. Midgut tissue of allatectomized roaches exhibited a significantly greater incorporation of isotope than that of sham-operated females.

4. The probable relationship between corpora allata and the synthesis of RNA, proteins, and uric acid during ovarian development has been discussed.

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INCORPORATION OF AMINO ACIDS INTO PROTEIN BY ARTIFICIALLY ACTIVATED NON-NUCLEATE FRAGMENTS OF SEA URCHIN EGGS

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Early experiments by Hultin (1950, 1952) and by Hoberman, Metz and Graff (1952) showed that fertilization results in a considerable increase in the incorporation of labeled constituents into protein by sea urchin eggs. That the increase was not due to an increase in permeability was shown by experiments (Nakano and Monroy, 1958) on preloading the eggs, before ovulation, with labeled amino acid and by later experiments (Hultin and Bergstrand, 1960) on cell-free amino acid incorporating systems. Tests (Hultin, 1961) in which there was exchange of ribosomes and supernatants of such systems prepared from fertilized and unfertilized eggs indicated that ribosomal inactivity was responsible for the low capacity of the unfertilized egg to incorporate amino acid into protein, a view that receives additional support from the finding (Monroy, Maggio and Rinaldi, 1965) that trypsin treatment can "activate" the ribosomes of unfertilized eggs. On the other hand, it has been shown (Tyler, 1962, 1963; Nemer, 1962; Nemer and Bard, 1963; Wilt and Hultin, 1962) that ribosomes from unfertilized eggs respond to the synthetic messenger RNA polyuridylic acid, and do so as effectively as do those from the fertilized eggs or later embryos. This could be interpreted to mean that the relative inertness of the unfertilized egg was due to a lack of messenger RNA which was then formed upon fertilization, presumably as a result of some stimulus to the zygote nucleus. For further exploration of this proposition non-nucleate fragments of sea urchin eggs were prepared and measurements made, on homogenates as well as on the intact cells, of the effect of parthenogenetic activation on their ability to incorporate amino acids into protein (Tyler, 1963; Denny and Tyler, 1964). This was found to be similar to that of the nucleate fragment or the fertilized whole egg. Independent autoradiographic studies of incorporation by artificially activated intact fragments (Brachet, Ficq and Tencer, 1963) gave similar results. Other experiments (Gross and Cousineau, 1963; Gross, 1964) showed that dactinomycin (actinomycin D), which inhibits DNA-primed RNA synthesis, fails to suppress the fertilization-induced activation of incorporation of amino acid into protein and does not block early development. Further it has been found (Maggio, Vittorelli, Rinaldi and Monroy, 1964) that RNA extracted from unfertilized eggs can act *in vitro* as a template for protein synthesis.

From these experiments it has been concluded that the mRNA for the protein synthesis of early development is present in the ripe unfertilized egg but in an inactive, or "masked," form. There is, however, also evidence (Nemer, 1963; Wilt, 1963, 1964; Glišin and Glišin, 1964; Gross, Malkin and Moyer, 1964; Gross, Kraemer and Malkin, 1965; Baltus, Quertier, Ficq and Brachet, 1965; Comb, Katz, Branda and Pinzino, 1965; Spirin and Nemer, 1965) that new RNA is synthesized during early development and that some of this is mRNA. It has been suggested on the basis of several lines of evidence (*cf.* Tyler, 1963, 1965) that this new mRNA, in turn, remains in a masked form, which may be represented by particles that have been termed informosomes (Spirin, Belitsina and Aitkhozhin, 1964; Spirin and Nemer, 1965), until a later stage of development. Further support of this view has been provided by electrophoretic studies (Spiegel, Ozaki and Tyler, 1965; Terman and Gross, 1966) showing that there are no marked qualitative differences in the pattern of soluble proteins that are formed during early development of sea urchin eggs and that development in the presence of dactinomycin did not detectably alter this pattern.

For further exploration of this problem the experiments with non-nucleate egg fragments have been extended to all 20 of the "coded" amino acids. In previous tests (Denny and Tyler, 1964; Baltus *et al.*, 1965) two different amino acids had been employed and the incorporation of each was stimulated by artificial activation of the fragment to an extent similar to that in the activated nucleated fragment or whole egg. The present experiments to which reference has been made earlier (Tyler, 1965) show this to hold for each of the 20 amino acids.

Materials and Methods

Eggs of the sea urchins *Lytechinus pictus* and *Strongylocentrotus purpuratus* were obtained by KCl-injection of the animals and handled in artificial sea water (Tyler and Tyler, 1966). The separation into non-nucleate and nucleate fragments was done by the centrifugation method on layers of sucrose-sea water of graded density, essentially as described by Harvey (1956). Eggs of different species may differ in density and other properties, as may also eggs of the same species at different times and different conditions of maintenance of the animals. The optimum conditions for producing non-nucleate fragments may change accordingly (*cf.* Costello, 1938). The following procedure was most often effective for the eggs of the two species that were used. Isotonic sucrose (1.1 molar) solution and sea water were mixed in various proportions and layered in 30-ml. plastic centrifuge tubes. For *L. pictus* eggs the percentage of sucrose solution, and the volume, of each layer, starting from the bottom, was 75% (5 ml.), 52% (5 ml.), 50% (5 ml.) 45% containing the eggs (15 ml.). Centrifugation was for 15 minutes at 10,000 rpm in the SW 25.1 rotor of the Spinco model L centrifuge. Generally the non-nucleate fragments banded sharply above the 75% layer while the unsplit eggs were in, or on, the 50% layer, and the nucleate fragments near the top of the 45% layer. However, the positions varied to some extent, evidently partly because of variation in the time after preparation at which the tubes were used. *S. purpuratus* eggs are denser and require stronger force for separation into the fragments. For these the sucrose-sea water layers were generally 80% (3 ml.), 75% (4 ml.), 66⅔% (10 ml.), 60% (10 ml.) and 50% containing the eggs (3 ml.). Centrifugation was for 5 minutes at 2500 rpm followed by 15 minutes at 10,000 rpm. The initial period at low speed served to stratify egg components so that the subsequent period of higher speed resulted in a more equal splitting into two fragments than if the initial period were omitted.

In most of the present experiments only the non-nucleate fragments of the centrifuged eggs were collected. This was usually done by puncturing the bottom of the centrifuge tube. Samples were examined to ascertain the absence of the nucleus and to measure the diameters. The fragments were generally uniform in size and had about half of the volume of the whole egg (average diameter = 112 μ for *L. pictus* and 78.4 μ for *S. purpuratus*).

For measurement of the incorporation of C^{14}-amino acid into protein the procedure of Mans and Novelli (1961), of plating the incubation mixtures on filter papers, was followed with some modifications (*cf.* Tyler, 1963; Tyler and Tyler, 1966). The filter papers ($1\frac{1}{2}'' \times 3''$, Whatman #1) were held with an intervening fold of aluminum foil at one end, in plastic clothespins mounted on a rack that supported also a manifold from which an airstream was directed to each paper. The quenched reaction mixtures (usually 0.30 ml.) were thus dried quickly after transfer to the papers, which were then dropped individually into a beaker of ice-cold 5% trichloroacetic acid (TCA). Generally not more than 2 dozen papers were processed together. However, larger numbers could be run in a single beaker without indication of transfer of radioactivity (tested by blank papers and by the t_0's). Further processing was with hot (90° C.) TCA, cool (20° C.) TCA, 95% ethanol, 100% ethanol, and ether, after which the papers (in the form of cylinders with non-overlapping edges) were transferred to the vials for determination of radioactivity by scintillation counting (Packard Tri-Carb Spectrometer).

The use of ice-cold TCA for the first treatment of the paper serves to reduce the amount of binding to the filter paper that occurs to various extent with the different amino acids, especially at elevated temperatures. Since in the present experiments whole eggs and intact fragments, rather than homogenates, were used these could be washed with sea water, after quenching with the C^{12}-amino acid, before transfer to the filter papers. This further reduced the opportunity for binding of the free C^{14}-amino acids to the filter paper. The values for the radioactivity of the t_0's (samples quenched with the C^{12}-amino acid solution at zero incubation time) in these experiments, were all very low and did not introduce a significant source of variation. The t_0's were mostly in the range of 0 to 60 cpm above background, and did not exceed 100 cpm in any of the tests except those with cystine, tryptophan and tyrosine, for which the values were in the range 50 to 150, 60 to 530 and 80 to 590, respectively.

For artificial activation of the non-nucleate fragments, and of the whole eggs, the single treatment with butyric acid was employed. An exposure of $1\frac{3}{4}$ minutes to a 0.005 molar solution proved to be optimum for both species. The procedure was to mix a small volume (usually 2 ml.) of the suspension with an equal volume of 0.01 molar butyric acid in a larger beaker and then add a large volume (usually 500 ml.) of sea water at the end of the treatment period.

Samples of the treated whole eggs and non-nucleate fragments were examined microscopically for determination of the per cent that were activated. As criteria of activation both membrane elevation and formation of the hyaline layer were used. The latter is particularly important for *L. pictus* since in this species the non-nucleate fragment may often fail to elevate a membrane on activation. Evidently in this species the separation of the fragments upon centrifugation causes a change (perhaps unrepaired rupture or loss) in the vitelline membrane such that there is no membrane elevation upon activation in most cases. Thus membrane elevation for the fragments of *L. pictus* eggs ranged around 15% to 25% in the present experiments. However, over-all activation was above 90% for both the fragments and the whole eggs in all the experiments reported here for both species. Since the errors in scoring are generally in the direction of counting activated eggs as unactivated, it may be assumed that activation was close to 100% in all experiments. In any case a scoring error of some 10% is less than many of the differences between duplicate samples that derive from sampling error and other sources.

At one-half hour after the butyric acid treatment, in the principal experiments, 0.25-ml. aliquots of the washed, and concentrated (generally about 10,000 to 20,000 per ml.), suspensions of the whole eggs and non-nucleate fragments were distributed (calibrated, wide-mouth, droppers) in duplicate to plastic tubes (15 mm. diameter) containing 0.05 ml. of the C^{14}-amino acid in sea water, and incubated at 20° C. with gentle shaking for one hour. The zero time (t_0) blanks for each C^{14}-amino acid were also set up in duplicate with a large excess of the corresponding C^{12}-amino acid present and kept in an ice bath for processing with the others. The t_0's received aliquots of the whole eggs.

Determinations of the quantity of egg material used in an experiment were done by counting the eggs in samples of the suspensions (*cf.* Tyler and Tyler, 1966) and by centrifugation in a Hopkins vaccine tube at 2500 *g* for 10 minutes. The average values obtained for the packed cell volumes for 10^4 eggs, were 0.0029 cc. for *S. purpuratus* and 0.0083 cc. for *L. pictus*. The volumes occupied by 10^4 eggs of these two species as calculated from their average diameters are 0.0025 cc. and 0.0075 cc.

EXPERIMENTS

Pattern of incorporation

Data for the incorporation of the 20 C^{14}-amino acids by activated whole eggs and activated non-nucleate fragments, in six experiments with *S. purpuratus* and six with *L. pictus*, are presented in Figures 1 and 2, respectively. In each experiment 10 amino acids were tested at one time with the whole eggs and non-nucleate fragments of the same batch of eggs. Comparisons may thus be made within each experiment, between whole eggs and non-nucleate fragments, on the basis of the pattern of incorporation of the 10 amino acids, apart from the absolute values, which involve the additional variation connected with determinations of egg quantity. Furthermore, in addition to its dependence on the kind of protein synthesized, the absolute value for incorporation of a particular C^{14}-amino acid also depends on the size of the intracellular pool of the corresponding C^{12}-amino acid, and this may vary from one batch of eggs to another.

In the data presented in Figures 1 and 2 the average values for the 10 different amino acids of an experiment are connected by broken lines. This was done so as to facilitate visualization of the pattern, the lines having no graphical significance. As the figures show, the patterns for the whole eggs and for the non-nucleate fragments are very much alike. Within each experiment whole eggs and non-nucleate fragments also show generally similar absolute values for incorporation of the individual amino acids. Occasional divergences that may be seen in one experiment are not observed in the other two of the same series. In certain of the

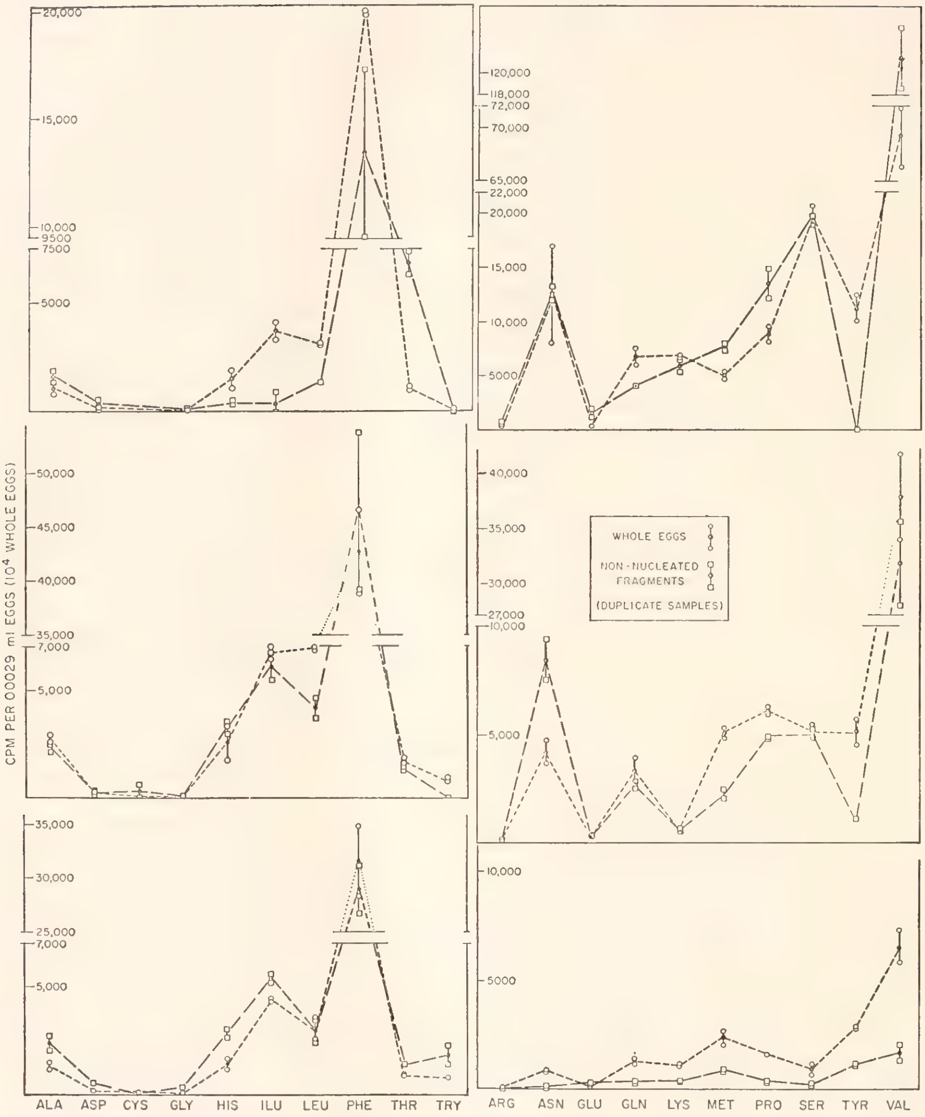

FIGURE 1. Incorporation of C^{14}-labeled amino acids into protein during one hour by non-nucleate fragments and whole eggs of *Strongylocentrotus purpuratus* at $\frac{1}{2}$ hour after activation with butyric acid. The individual amino acids were tested in groups of 10 in each experiment in duplicate samples of whole eggs and of non-nucleate fragments, and at 0.4 μc./ml. Three experiments with one group are presented in the three charts on the left and three with the other group on the right. Specific activities in cpm and names for abbreviations are: 4.24 (Alanine), 22.5 (Aspartic Acid), 7.70 (Cystine), 5.25 (Glycine), 22 (Histidine), 6.16 (Isoleucine), 6.3 (Leucine), 360 (Phenylalanine), 5.15 (Threonine), 8.95 (Tryptophan), 9.23 (Arginine), 22.5 (Asparagine), 205 (Glutamic Acid), 4.05 (Glutamine), 7.1 (Lysine, except 43.8 in upper right chart), 15.6 (Methionine), 7.1 (Proline), 1.7 (Serine), 11.54 (Tyrosine) and 200 (Valine). For the purpose of enabling the pattern of incorporation to be more readily visualized the averages are connected by dashed lines. The lines have no other significance.

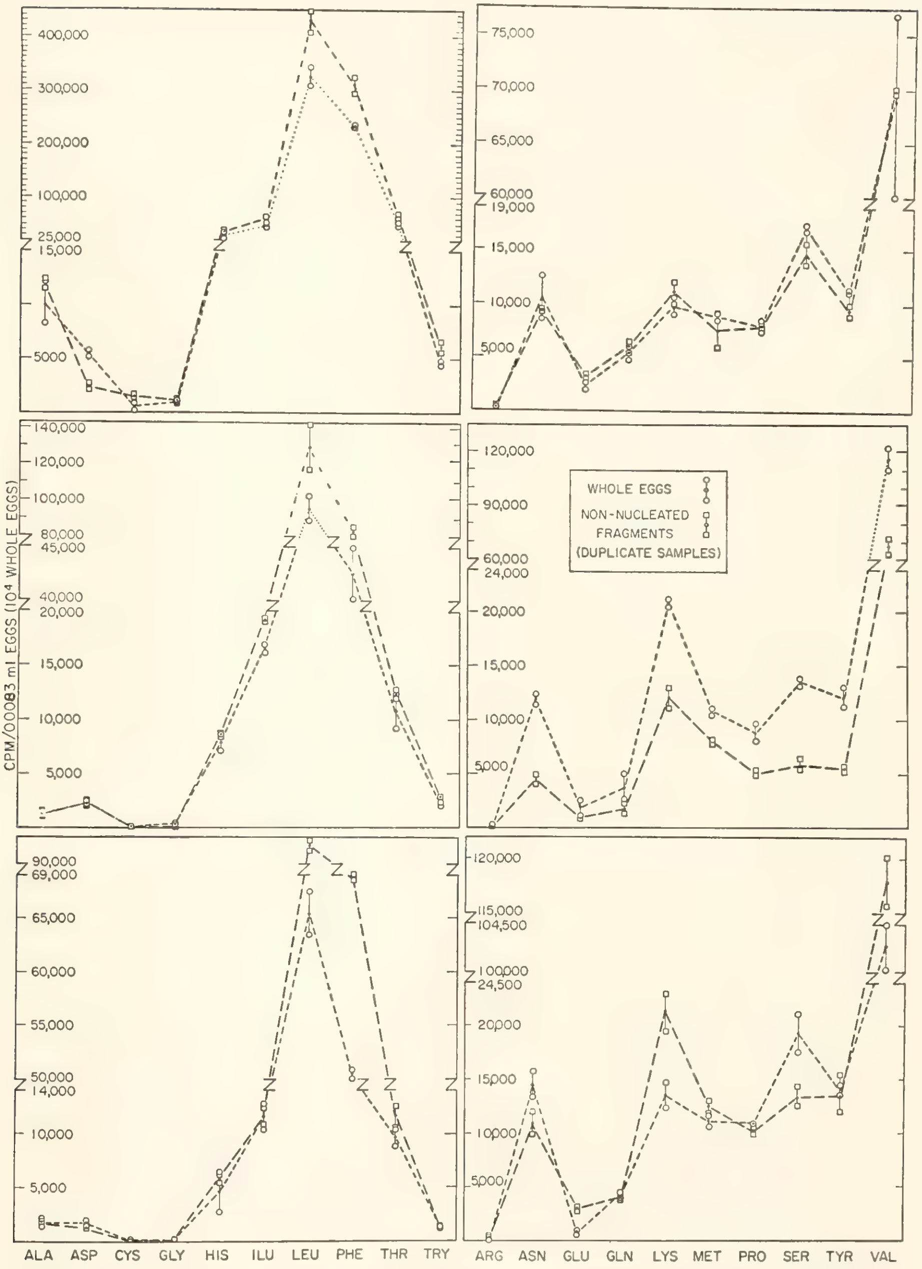

FIGURE 2. Same as Figure 1 but for eggs of *Lytechinus pictus* and with following differences in specific activities: 160 (Leucine), 25.6 (Threonine) and 43.8 (Lysine).

experiments, *e.g.* the middle one on the right of Figure 2, the absolute values for each of the 10 amino acids are lower in the non-nucleate fragments than in the whole eggs. This doesn't hold for the other two experiments with this set of amino acids, nor with the three involving the same set of amino acids in the other species. It does not, then, indicate a significantly lower capacity of the non-nucleate fragment for

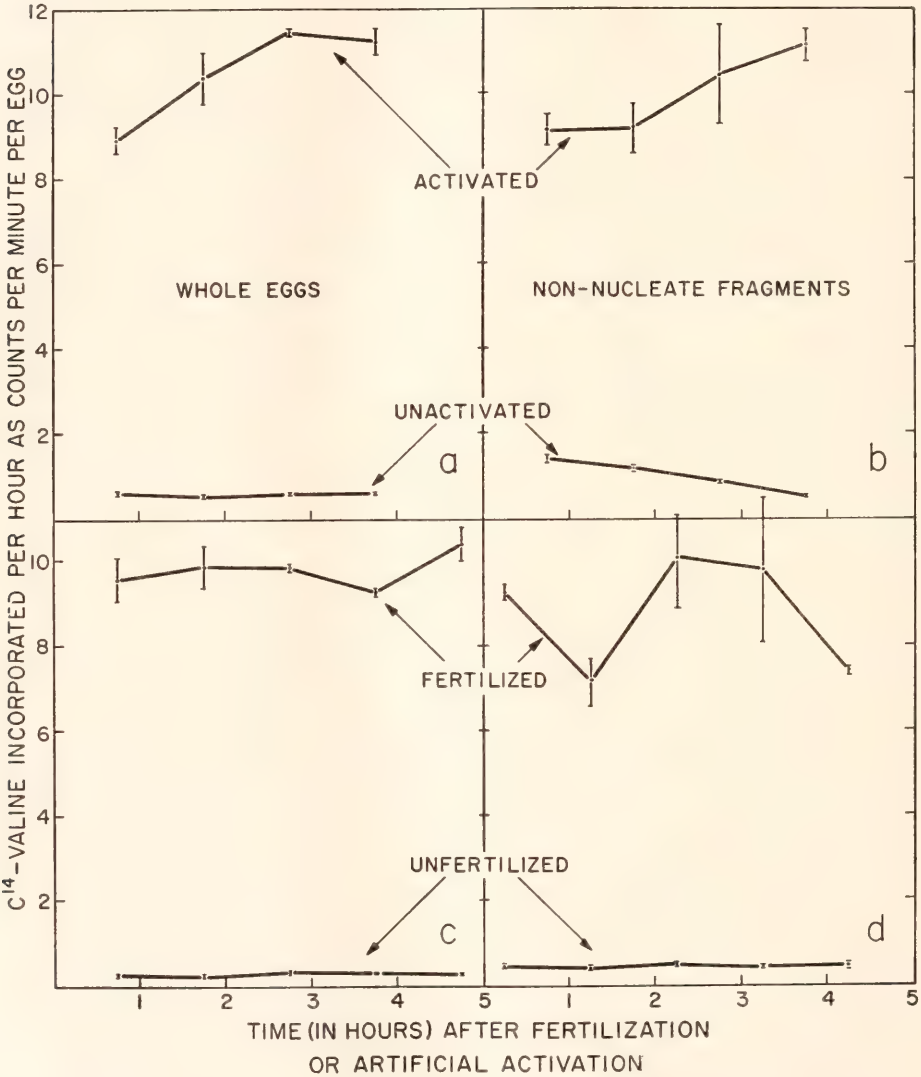

FIGURE 3. C^{14}-valine incorporated into protein in one hour by eggs of *Lytechinus pictus* starting at the indicated times. *a* and *b*, whole eggs and non-nucleate fragments of the same experiment. *c* and *d*, two separate experiments. Incubations at 20° C. in 0.3 ml. of C^{14}-valine (sp. act. 200 cpm) in sea water at 0.4 μc./ml. and at egg concentrations near 1% (3600 eggs) by volume. Activation with butyric acid as described. Vertical lines show spread of duplicates.

incorporation of the amino acids into protein. Most probably, in this experiment, the errors in estimation of egg quantity and in percentage activation were relatively greater than other sources of variation.

If the data are normalized with respect to any one amino acid, and account is taken of the fact that specific radioactivities differed in certain cases, it appears that the two species differ somewhat in pattern of incorporation. The non-nucleate fragments are similar to the whole eggs in this respect and also with respect to the absolute values for each amino acid.

Time course of incorporation

The incorporation of C^{14}-valine into protein by non-nucleate fragments and whole eggs of *Lytechinus pictus* was measured at various times during the first five hours after artificial activation. Data of one experiment are presented in Figure 3a and b, which includes also the unactivated controls. Data for two separate experiments with fertilized eggs, and their unactivated controls, are shown in Figure 3c and d.

From the results of this and another similar experiment it appears that the incorporation of C^{14}-valine by the activated non-nucleate fragment remains as high as that of the activated whole egg during this period of time. The small rise that appears in the data of Figure 3a and b is probably of no significance since it is not evident in the other experiment. The same may be said for the decrease with time shown by the control, unactivated, non-nucleate fragments.

Figure 3 also shows an approximate constancy of incorporation of the C^{14}-valine into protein on the part of the fertilized eggs during this period. The initial great increase in incorporation into protein occurs very soon after fertilization and no significant further increase appears during the first five hours.

In the experiment plotted in Figure 3c the eggs were distributed to the incubation tubes at one time and the C^{14}-valine added at the indicated time. In that of Figure 3d the eggs were distributed at the indicated times to the tubes. This difference in procedure probably accounts for the larger fluctuations obtained in the latter experiment.

DISCUSSION

The present experiments show that the previously reported similarity of artificially activated non-nucleate fragments to artificially activated (or fertilized) whole eggs with respect to the incorporation of amino acid into protein, applies to all 20 of the coded amino acids. In individual experiments in which sets of 10 amino acids were tested at the same time the relative incorporation values for the non-nucleate fragments exhibit a pattern very similar to that of the whole eggs. Also the absolute values are similar. The use of two different species of sea urchins, differing somewhat in pattern of incorporation and absolute values, facilitated the comparisons. Further, in experiments in which incorporation of one of the amino acids was followed for several hours the non-nucleate fragment remained as active as the whole egg.

It may be concluded that the kinds of protein synthesized by the activated non-nucleate fragment do not differ appreciably from those of the whole egg during early development. The previous experiments with non-nucleate fragments, the experiments with dactinomycin, and the demonstration of template activity of RNA extracts from unfertilized eggs, to which reference was made in the introduction, provided evidence that the cytoplasm of the unfertilized egg contains masked messenger (m)RNA that becomes active upon fertilization. The present experiments indicate also that it is this mRNA that serves for the bulk of protein synthesis in early development.

The data of Figure 3 show that, following the great increase shortly after fertilization, there is relatively little change in rate of incorporation of amino acid into protein during the first few hours of development. The occurrence, during early development, of a plateau in the rate of incorporation of amino acid into protein by sea urchin embryos has been reported by several other investigators (*e.g.,* Hultin, 1952, 1961; Markman, 1961; Giudice, Vittorelli and Monroy, 1962; Gross and Cousineau, 1964; Berg, 1965; *cf.* Monroy, 1965), although the reports differ to some extent in the time at which this occurs. One may infer that the mRNA molecules are all, or mostly all unmasked at the time that the plateau occurs. From the present data it appears that the complete unmasking occurs very soon after fertilization. These inferences presuppose that the mRNA remains stable during early development, an assumption that seems to accord with the results of experiments on the mRNA of other animal cells (*cf.* Marks, Burka, Conconi, Perl and Rifkind, 1965).

One may also infer from these results that if any significant amount of new mRNA is formed in early development it must remain largely inactive during this period. As stated in the introduction, convincing evidence has, in fact, been accumulating that new mRNA is produced during early cleavage stages of sea urchins. It was also surmised that this mRNA for the most part, remains masked until a later stage of development. There is now evidence (Spirin and Nemer, 1965) that the mRNA synthesized during early development of sea urchins occurs in the form of RNP particles, that are lighter than the ribosomes and also in light polyribosomes, neither of which are active in the incorporation of amino acid into protein. The latter activity is exhibited mainly by the heavy polyribosomes which do not become labeled by an RNA precursor administered during early development.

Reference was also made in the introduction to two electrophoretic studies (Spiegel *et al.,* 1965; Terman and Gross, 1966) showing no marked qualitative differences in the soluble proteins that are formed during early development. In the study by Terman and Gross (1966), in addition to the similarity in radioactivity pattern for newly synthesized proteins in control and actinomycin-treated embryos during the period of cleavage, systematic changes were also noted. These appeared in the autoradiograms from blastula extracts and became more obvious at gastrulation. Terman and Gross interpret this to imply either differences in the kinds of synthesized proteins or changes in rates of synthesis of species in the same set. Dactinomycin-treated embryos follow the controls with regard to these changes, which cannot, then, be attributed to the new mRNA that is synthesized during early development. Possibly, then, while the major portion of the masked mRNA present in the unfertilized egg is activated shortly after fertilization, there may be some selective activation of some masked messengers at later stages. In an initial investigation (Spiegel and Tyler, 1966) of this proposition, with micromeres, prepared from sea urchin eggs at the 16-cell stage, incorporation of amino acid into protein was found to be quantitatively similar to that of the other cells.

Apart from the question of selective activation of various kinds of masked mRNA it appears that the production of mRNA in an initially inactive form occurs commonly, or perhaps invariably, during development. It has been previously indicated (Tyler, 1965), on the basis of a survey of experiments on the time of occurrence of dactinomycin sensitivity in the differentiation of a particular tissue, that the production of masked mRNA's represents the "determination process" in embryonic development. Actual differentiation, then, follows a later unmasking process which very likely acts at one time, or within a short period of time, on all the species of mRNA molecules involved in the differentiation of the tissue.

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SUMMARY

1. Artificially activated non-nucleate fragments and whole eggs of the sea urchins *Strongylocentrotus purpuratus* and *Lytechinus pictus* have been compared with regard to their ability to incorporate C^{14}-labeled amino acid into protein. Each of the 20 "coded" amino acids has been tested, the tests being done in groups of ten. In both species non-nucleate fragments closely resemble whole eggs with regard to the pattern of incorporation of the sets of ten amino acids. Also there is similarity in the absolute incorporation values when the comparisons are made within individual experiments in which variation in size of the endogenous amino acid pool is not a factor.

2. The similarity in amino acid (valine) incorporation is maintained during the first six hours after activation. The values are similar to those for fertilized eggs, as earlier studies had also shown. Further, following the initial great rise shortly after fertilization, or artificial activation, the rate of incorporation remains relatively constant during this period.

3. The results provide additional evidence that the activation of protein synthesis upon fertilization does not depend upon production of mRNA by the nucleus. Further they show that the activated non-nucleate fragment and the whole egg do not differ appreciably in the kinds of proteins they synthesize during early development, that the corresponding mRNA's are all, or mostly all present in the unfertilized egg, and that most or all of these become "unmasked" soon after fertilization or artificial activation.

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MATING BEHAVIOR AND SPERMATOPHORE MORPHOLOGY IN THE PSEUDOSCORPION DINOCHEIRUS TUMIDUS BANKS (CHELIFERINEA, CHERNETIDAE)[1]

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Recent studies on the mating behavior and spermatophore morphology of some European pseudoscorpions (Weygoldt, 1966) have shown a variety of methods of sperm-transfer in this group. All male pseudoscorpions deposit spermatophores from which the females take up sperm. But the means by which sperm-transfer is ensured differ among families and genera. In the more primitive families Chthoniidae, Neobisiidae, Garypidae and Cheiridiidae there is no mating; the females trace the spermatophores chemotactically. The Chernetidae and Cheliferidae, on the other hand, have a courtship dance during which the male deposits spermatophores and leads the female to them. The comparison of different methods of sperm-transfer in pseudoscorpions has led to the assumption of an evolution from simple behavior without mating (*e.g.,* Chthoniidae) to the most complex mating dances (*i.e,* Cheliferidae). To confirm this assumption, further knowledge of sperm-transfer methods in different species of pseudoscorpions seems necessary. In this paper the mating dance and spermatophore of *Dinocheirus tumidus* Banks are described. This species is of special interest because of its sexual dimorphism. The pedipalpal hand of the male is much stouter than that of the female, being greatly swollen on the inner and dorsal side. It is not known how the male uses this feature.

MATERIAL AND METHODS

The animals were collected at Big Pine Key, Florida, at the end of December, 1965. They occurred in great numbers under pieces of wood in the beach drift line. I collected nearly 30 specimens; only six were females. They were kept in the laboratory with the methods described by Weygoldt (1966). Males and females were maintained separately. Shortly after capture some males were placed with females and mating was observed almost immediately. Their behavior was studied with a dissecting microscope. After a few days all females were pregnant and no further mating was observed.

RESULTS

When males are placed with females they walk around and attack all members of their own species. When one encounters another male, a struggle begins in which each male tries to grasp one or both palpal hands of the other. Then, they pull each other as strongly as possible. This may last for several minutes. Finally,

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they separate and one of them quickly runs backward, showing excited beckoning movements of his pedipalps. Sometimes he is followed a short distance by the other.

The mating behavior

When a male encounters a female, he grasps one of her palpal hands. Sometimes the female tries to escape; then he rapidly grasps both of her hands and pulls her strongly several times. But soon he releases her second hand and holds, for instance, her right hand in his left hand. This is maintained throughout the whole mating dance, during which several spermatophores are deposited and transferred. At the beginning the male sometimes walks around and pulls the female behind him.

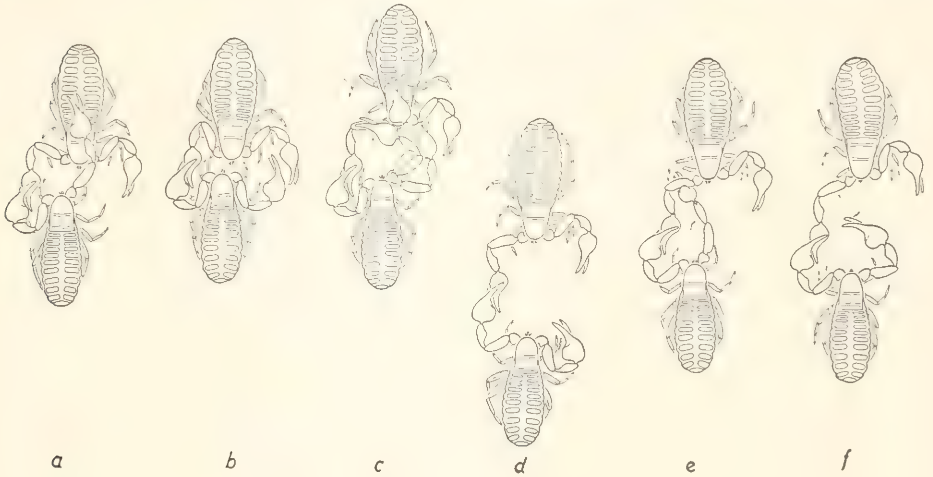

FIGURE 1. The mating dance of *Dinocheirus tumidus*. a: The male (below) touches the back of the female. b: The male begins to deposit the spermatophore. c: The male forms the sperm package of the spermatophore and touches the female with characteristic quick movements of his free pedipalp. d: The male pulls the female towards the spermatophore. e: The male has pushed back the female and now shakes her. He pulls one palpal hand of the female and presses his free hand against the femur of the same female pedipalp. Between mates the stalk of the spermatophore is visible. f: The male pushes down the stalk of the spermatophore. The female bends her opisthosoma and presses her ventral side against the ground, thereby removing the sperm package from her genital opening.

After this he places himself right before the female and the mating dance begins. The male slowly nears the female; she slowly steps backward. Then, he pulls her, forcing her near him, but she again slowly steps backward. Suddenly the male extends his free pedipalp and touches the back of the female with his hand (Fig. 1a). The female immediately stops stepping backward. Now the male shows very characteristic movements with his free pedipalp. He moves his hand above the female and with his fingers touches her back, the upper side of the femur of one of her pedipalps (the hand of which he holds in his other hand), and the under side of her other pedipalpal femur (Fig. 1c). At the same time he steps forward, nearing the female. The female lifts her body and finally stands on extended legs before the male. The male now stops moving his free pedipalp and deposits the spermatophore on the substratum (Fig. 1b, 2a). At first, when he is secreting the stalk of the spermatophore, the male does not show any movement, and the female begins to step backward. But during formation of the sperm package the male again touches the female. The movements of his free pedipalp become very quick and excited (Fig. 1c). Suddenly he stops. The spermatophore is now formed and the male gradually steps backward over it and pulls the female to it (Fig. 1d). The female follows, at first reluctantly, and then slowly steps over the spermatophore. When her genital opening is just above the spermatophore she takes up the sperm package. At the same moment the male pushes her back with his free hand; the spermatophore stalk without sperm package now becomes visible between mates. Then, he brings his free hand under the femur of her pedipalp which he holds in his other hand. He presses the swollen inner side of his free hand against this femur (Fig. 1e). With one hand pulling the female's hand and with his other

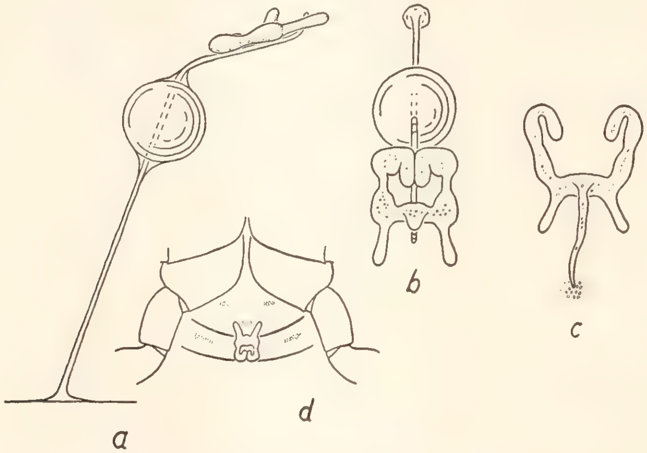

FIGURE 2. The spermatophore of *Dinocheirus tumidus.* a: The spermatophore seen from the side. b: The spermatophore seen from above. c: The sperm package after it is put into water. d: The female genital field with the sperm package. Only the duct of the sperm package has entered the genital opening.

hand pushing the female's femur of the same pedipalp, the male shakes the female with a series of short and quick movements forward and backward.

After these shaking movements the first sperm transfer is finished. The male removes his free hand from the female pedipalp femur and searches for the spermatophore stalk which is still standing between mates. When he finds it he pushes it against the ground (Fig. 1f). Sometimes he does not find it, because it was thrown down by the legs of the female or by some other accident. Then, he pushes other particles to the side that lay between him and the female (*e.g.*, pieces of sand).

During the same time the female moves her opisthosoma. She bends it to the sides and up and down. Especially she presses her ventral side against the ground several times and thereby removes the emptied sperm package from her genital opening (Fig. 1f).

Following this the male again touches the back of the female and the whole process is repeated. The mating dance may last 15 to 30 minutes, and every five to seven minutes a new spermatophore is deposited and transferred.

At the end of the mating dance both mates lie down and rest for 10 to 15 minutes without moving, the male still holding one hand of the female. After this repose they may begin a new mating dance or the female may try to escape, vigorously attacking the male with her free hand until the male releases her other hand. Then, they separate and the female hurries away.

Sometimes the female is not receptive when the first or even the second spermatophore is deposited. In this case the male does not succeed in pulling her over the spermatophore and she does not take up the sperm package. Then, the male does not press the swollen inner side of his free hand against the female pedipalpal femur and does not shake the female. If, after depositing the spermatophore, he does not succeed in pulling the female over it, he waits a short time and then carefully searches and destroys the spermatophore by pushing it down. He then continues the mating dance.

The spermatophore

The spermatophore consists of a stalk, a drop of fluid, and the sperm package (Fig. 2a). The stalk is attached to the ground with a small base. It is about 0.5 mm. high. Since the male moves backward a little when he secretes the spermatophore, the stalk is inclined. Its upper part is bent backward and is nearly horizontal. This horizontal part of the stalk bears the sperm package. Under the sperm package there is a large drop of fluid.

The sperm package consists of two tubes which open into a common duct (Fig. 2b, c). The blind ends of the tubes lie at the anterior part of the spermatophore and are bent backward. From this anterior end the tubes turn to the sides and then backward, joining at the posterior end of the spermatophore. A short distance before their junction each tube has a side branch extending posteriorly. In the newly deposited spermatophore the common duct of the sperm package is short and closed. When the sperm package is put into water, water enters it through the wall, triggering a swelling mechanism. Very quickly the duct elongates until it becomes four to five times longer. At the same time the swelling mechanism inflates the tubes. Now, the sperm package looks like an H, the duct rising in the middle of the crossbeam. Finally the duct opens and the sperm are forced out.

During sperm transfer, when the female takes up the sperm package, only the duct of the package enters the female genital opening, the remaining part of the package staying outside (Fig. 2d). Simultaneously, the drop of fluid is pushed between the tubes of the sperm package. Together with the fluid inside the genital atrium of the female, it triggers the swelling mechanism which elongates the duct and forces the sperm out of the sperm package. The sperm presumably go directly into the receptaculum seminis of the female.

DISCUSSION

Until now the reproductive behavior of two species of Chthoniidae, one species of Neobisiidae, two species of Cheiridiidae, four species of Chernetidae, and two species of Cheliferidae has been studied. The males of the Chthoniidae, Neobisiidae and Cheiridiidae (Kew, 1930; Weygoldt, 1966) deposit spermatophores without mating, even when females are not present. The females trace the spermatophore chemotactically. In these groups sperm-transfer is ensured by the great numbers of spermatophores deposited. In the two observed species of *Chthonius, Chthonius tetrachelatus* and *Chthonius ischnocheles,* and in the *Cheiridiidae, Cheiridium museorum* and *Apocheiridium ferum,* the males not only deposit spermatophores, but also destroy the old ones by pushing them down. In most cases they destroy an old spermatophore before depositing a new one. In both cheiridiids, the females also destroy old spermatophores.

The Chernetidae and Cheliferidae, on the other hand, have a complex mating behavior (Kew, 1912; Vachon, 1938; Weygoldt, 1966). The chernetid male grasps one or both palpal hands of the female and after having made some characteristic movements (*e.g.*, stepping forward and backward several times), he deposits the spermatophore and pulls the female to it. The mating dance of the Cheliferidae is more complicated. The male dances without touching the female, and by following his movements she finds the spermatophore. After this the male grasps the female pedipalpal femora with his hands and with his forelegs assists her in taking up the sperm. In this group a close contact between mates is not established before the spermatophore has been deposited.

Dinocheirus is a chernetid and its mating dance is of the chernetid type. But the display shown by the male is quite different from those of the males of other species investigated. In *Chernes cimicoides, Chernes hahni,* and *Lasiochernes pilosus* the male grasps both hands of the female and walks forward and backward with her several times before depositing the spermatophore. Sometimes the male of *Chernes cimicoides* only grasps one hand of the female, but even then he does not show any display with his free hand. Instead, he shows characteristic movements with his first pair of legs, touching the female with it. In *Dendrochernes cyrnus* (Kew, 1912), the male holds only one hand of the female and strongly displays with his free pedipalp and first pair of legs. He moves his pedipalp in a "remarkable threatening or perhaps beckoning manner." In this respect there is some similarity between the mating dances of *Dendrochernes* and *Dinocheirus.* But the way in which the male of *Dinocheirus* touches the female before and during the formation of the spermatophore, and, further, the way he treats her afterward with the swollen inner side of his hand are remarkable and bear no similarity to the behavior of the other species. An obvious display after sperm transfer has been reported only for *Chernes cimioides* (Weygoldt, 1966), the male of which slowly "tries to climb" on the back of the female. But this is not comparable to the way the male of *Dinocheirus* shakes the female, at the same time pushing and pulling one of her pedipalps. In the other species both mates rest without movement after the female has taken up the sperm package.

It should be mentioned that there are some species in other genera with a similar sexual dimorphism, for instance *Hesperochernes molestus.* It would be interesting to know whether these species show a similar display with their hand. It would also be of interest to know how the male of *Mirochernes dentatus* makes use of the anvil-shaped process on the inner surface of his pedipalpal hand.

Another remarkable feature of the mating dance of *Dinocheirus* is the way the male destroys whole spermatophores or the remaining stalk. This behavior has not been observed in any other chernetid or cheliferid. It resembles in some respects the behavior of some chthoniids and cheiridiids where the male often destroys an old spermatophore before forming a new one.

Also remarkable is the time used for one sperm transfer. In *Chernes* and *Lasiochernes* 10 to 30 minutes are required for the whole process, in *Dinocheirus* only five to seven minutes.

The spermatophore of *Dinocheirus* is of the chernetid type too. At first it seems to be different, but that is only because of the side branches extending posteriorly on the sperm package. The only remarkable difference is the small size of the spermatophore. In *Chernes* and *Lasiochernes* the sperm package is so big that it covers the whole genital field of the female when it is taken up. The sperm package of the *Dinocheirus* spermatophore is much smaller and contains less sperm. Therefore, the female has to take up several sperm packages to fill her receptaculum seminis.

I thank Dr. W. B. Muchmore, University of Rochester, for the determination of the species.

Summary

1. The mating behavior and the spermatophore of *Dinocheirus tumidus* Banks are described.

2. During the mating dance the male grasps one palpal hand of the female. Then, he shows a characteristic display with his other hand, touching the back of the female. Finally he deposits a spermatophore and pulls the female over it. When she has taken up the sperm package, the male presses the swollen inner side of his free hand against the femur of one of the female pedipalps (the hand of which he holds in his other hand) and shakes the female with a series of short, quick movements.

3. The spermatophore is of the chernetid type. It consists of a stalk, a drop of fluid and a sperm package. Only the duct of the sperm package is introduced into the female genital opening. Then, the sperm package is emptied by a swelling mechanism triggered by the drop of fluid.

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